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ABSTRACT

These instructor plans of instruction and lesson plans and student text and review materials for a secondary-postsecondary-level course for medical laboratory specialist are one of a number of military-developed curriculum packages selected for adaptation to vocational instruction and curriculum development in a civilian setting. It is the second of two courses (see Note) designed to provide the necessary knowledge and techniques to perform basic procedures in a medical laboratory. The outline of instruction suggests number of hours of class time devoted to each of four subject areas, a total of 132 hours of instruction: Basic Procedures in Urinalysis (7 lessons, 48 hours), Basic Procedures in Serology (7 lessons, 24 hours), and Basic Procedures in Parasitology (19 lessons, 60 hours). Instructor materials include outlines of the subject areas and lesson plans listing objectives, texts, references, tools and equipment, training aids, and lecture outline. Student materials include a workbook on basic medical parasitology and text materials from the Army Training Manual 8-227. (YLB)

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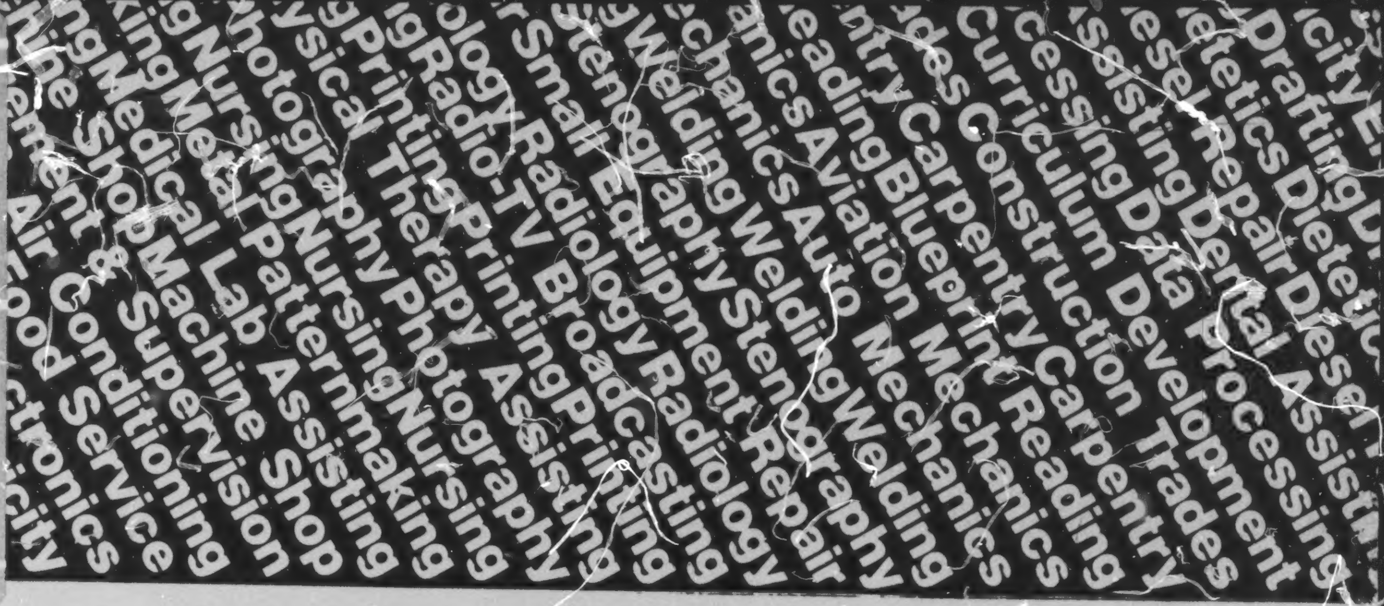
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Military Curricula for Vocational & Technical Education

MEDICAL LABORATORY SPECIALIST
(BASIC) PART II 10-15

CE 024 390

U.S. DEPARTMENT OF HEALTH
EDUCATION & WELFARE
NATIONAL INSTITUTE OF
EDUCATION

THE NATIONAL CENTER
FOR RESEARCH IN VOCATIONAL EDUCATION
THE OHIO STATE UNIVERSITY

This military technical training course has been selected and adapted by The Center for Vocational Education for "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education," a project sponsored by the Bureau of Occupational and Adult Education, U.S. Department of Health, Education, and Welfare.

MILITARY CURRICULUM MATERIALS

The military-developed curriculum materials in this course package were selected by the National Center for Research in Vocational Education Military Curriculum Project for dissemination to the six regional Curriculum Coordination Centers and other instructional materials agencies. The purpose of disseminating these courses was to make curriculum materials developed by the military more accessible to vocational educators in the civilian setting.

The course materials were acquired, evaluated by project staff and practitioners in the field, and prepared for dissemination. Materials which were specific to the military were deleted, copyrighted materials were either omitted or approval for their use was obtained. These course packages contain curriculum resource materials which can be adapted to support vocational instruction and curriculum development.

The National Center Mission Statement

The National Center for Research in Vocational Education's mission is to increase the ability of diverse agencies, institutions, and organizations to solve educational problems relating to individual career planning, preparation, and progression. The National Center fulfills its mission by:

- Generating knowledge through research
- Developing educational programs and products
- Evaluating individual program needs and outcomes
- Installing educational programs and products
- Operating information systems and services
- Conducting leadership development and training programs

FOR FURTHER INFORMATION ABOUT
Military Curriculum Materials
WRITE OR CALL
Program Information Office
The National Center for Research in Vocational Education
The Ohio State University
1960 Kenny Road, Columbus, Ohio 43210
Telephone: 614/486-3655 or Toll Free 800/848-4815 within the continental U.S. (except Ohio)

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Military Curriculum Materials for Vocational and Technical Education

Information and Field Services Division

The National Center for Research in Vocational Education

Military Curriculum Materials Dissemination Is . . .

. . . an activity to increase the accessibility of military-developed curriculum materials to vocational and technical educators.

This project, funded by the U.S. Office of Education, includes the identification and acquisition of curriculum materials in print form from the Coast Guard, Air Force, Army, Marine Corps and Navy.

Access to military curriculum materials is provided through a "Joint Memorandum of Understanding" between the U.S. Office of Education and the Department of Defense.

The acquired materials are reviewed by staff and subject matter specialists, and courses deemed applicable to vocational and technical education are selected for dissemination.

The National Center for Research in Vocational Education is the U.S. Office of Education's designated representative to acquire the materials and conduct the project activities.

Project Staff:

Wesley E. Budke, Ph.D., Director
National Center Clearinghouse

Shirley A. Chase, Ph.D.
Project Director

What Materials Are Available?

One hundred twenty courses on microfiche (thirteen in paper form) and descriptions of each have been provided to the vocational Curriculum Coordination Centers and other instructional materials agencies for dissemination.

Course materials include programmed instruction, curriculum outlines, instructor guides, student workbooks and technical manuals.

The 120 courses represent the following sixteen vocational subject areas:

- Agriculture
- Aviation
- Building & Construction Trades
- Clerical Occupations
- Communications
- Drafting
- Electronics
- Engine Mechanics
- Food Service
- Health
- Heating & Air Conditioning
- Machine Shop
- Management & Supervision
- Meteorology & Navigation
- Photography
- Public Service

The number of courses and the subject areas represented will expand as additional materials with application to vocational and technical education are identified and selected for dissemination.

How Can These Materials Be Obtained?

Contact the Curriculum Coordination Center in your region for information on obtaining materials (e.g., availability and cost). They will respond to your request directly or refer you to an instructional materials agency closer to you.

CURRICULUM COORDINATION CENTERS

EAST CENTRAL
Rebecca S. Douglass
Director
100 North First Street
Springfield, IL 62777
217/782-0759

MIDWEST
Robert Patton
Director
1515 West Sixth Ave.
Stillwater, OK 74704
405/377-2000

NORTHEAST
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Director
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Trenton, NJ 08625
609/292-6562

NORTHWEST
William Daniels
Director
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Airdustrial Park
Olympia, WA 98504
206/753-0879

SOUTHEAST
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Mississippi State University
Drawer DX
Mississippi State, MS 39762
601/325-2510

WESTERN
Lawrence F. H. Zane, Ph.D.
Director
1776 University Ave.
Honolulu, HI 96822
808/948-7834

MEDICAL LABORATORY SPECIALIST II | Classroom Course 10-15

311-92B10

Developed by:
United States Army

Development and Review Dates:
May 1975

Occupational Area:
Health

Target Audiences:
Grade 11 - Adult

Print Pages: 445

Microfiche: 8

Availability:
Vocational Curriculum Coordination Centers

Contents:	Type of Materials: Lesson Plans:	Programmed Text:	Student Workbook:	Handouts:	Text Materials:	Audio-Visuals:	Instructional Design: Performance Objectives:	Tests:	Review Exercises:	Additional Materials Required:	Type of Instruction: Group Instruction:	Individualized:
Annex F - Basic Procedures in Urinalysis	●[1]				●		●			x	●	
Annex G - Basic Procedures in Serology	●			●	●	x	●	●		x	●	
Annex H - Basic Procedures in Parasitology	●		●		●	x	●			x	●	

[1] Lesson outline only

X Materials are recommended but not provided.

THE NATIONAL CENTER FOR RESEARCH IN VOCATIONAL EDUCATION
OSU The Ohio State University
1960 Kenny Road
Columbus, Ohio 432
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Course Description:

This course is designed to provide the necessary knowledge and techniques to perform basic procedures in a medical laboratory related to urinalysis, serology, parasitology. The course consists of 3 subject areas requiring 132 hours of instruction. Annex A-E are included in a previous course, Medical Laboratory Specialist I.

Annex F - Basic Procedures in Urinalysis contains 7 lessons covering 48 hours of instruction.
- Introduction (2 hours)
- Physical Properties of Urine (2 hours)
- Chemical and Microscopic Examination (2 lessons, 6 hours)
- Urinalysis Techniques (36 hours)
- Exam (2 hours)

Annex G - Basic Procedures in Serology consists of 7 lessons covering 24 hours of instruction.
- Introduction (2 hours)
- Immunological Tests (1 hour)
- Syphilis Tests (3 hours)
- Pregnancy Tests (1 hour)
- Serological Techniques (10 hours)
- Exams (2 lessons, 7 hours)

Annex H - Basic Procedures in Parasitology contains 19 lessons covering 60 hours of instruction.
- Introduction (1 hour)
- Laboratory Techniques (3 lessons, 4 hours)
- Protozoan Organisms (4 lessons, 12 hours)
- Stool Specimen (1 lesson, 1 hour)
- Gresma Stain (2 lessons, 3 hours)
- Malaria Organisms (1 lesson, 2 hours)
- Helminths (1 lesson, 1 hour)
- Other Parasitic Organisms (1 lesson, 2 hours)
- Exams (4 lessons, 7 hours)

The course contains both teacher and student materials. The printed teacher material includes lesson objectives and lesson outlines describing references, aids and equipment, lesson content. Student materials include a workbook on basic medical parasitology, and text materials from Army Training Manual 8-227 on the topics.

MEDICAL LABORATORY SPECIALIST (BASIC)

PART II

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SECTION IV - ANNEXES

ANNEX F

BASIC PROCEDURES IN URINALYSIS

OBJECTIVE: Given simulated urine specimens, perform routine urinalysis procedures, to include tests for physical, chemical, and microscopic properties with an acceptable degree of accuracy.

Subject and LP No	Lesson Objectives	References
Introduction to Urinalysis 08-280-801	Given a diagram, identify the major macroscopic and microscopic anatomical structures of the urinary tract. Given a specific anatomical region of the nephron, state its function. Name the types of urine specimens commonly collected for laboratory study and specify reasons for their collection. Given a type of urine specimen, state the reason and what method of preservation should be used.	TM 8-227-6, Intro to Part III; Kark, pp 1-5; Lippman, chap 3.
Physical Properties of Urine 08-280-803	Identify procedural steps involved in performing tests for physical properties of urine, to include color, appearance, volume, pH, and specific gravity.	TM 8-227-6, chap 16; Kark, pp 8-13; Lippman, chap 3.
Chemical Examination of Urine 08-280-806	Identify procedural steps involved in performing qualitative and/or semi-quantitative tests for chemical constituents of urine, to include protein, reducing sugars, ketones, bilirubin, blood urobilinogen, and calcium.	TM 8-227-6, chap 18; Kark, pp 18-56; Lippman, chap 3.
Microscopic Examination of Urinary Sediment 08-280-810	Identify procedural steps involved in preparing urine specimens for microscopic examinations. Identify procedural steps involved in performing microscopic examination of urinary sediment. Identify objects commonly seen in normal and abnormal urine sediment, to include blood cells,	TM 8-227-6; chap 17, pp 17-1 thru 17-15; Kark, pp 60-75; Lippman, chap 1.

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Subject and LP No	Lesson Objective	References
	epithelial cells, casts, crystals, miscellaneous structures, and artifacts, and cite reporting nomenclature commonly used for each object.	
Urinalysis Techniques 08-280-812	Given known urine specimens, perform qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood, calcium, color appearance, pH, and specific gravity along with proper usage of reporting nomenclature as given in class. Given known urine specimens, detect and identify structures commonly seen in normal and abnormal urine sediment, miscellaneous structures and artifacts along with proper usage of reporting nomenclature as given in class.	All previous references.
Urinalysis Techniques 08-280-813	Given simulated urine specimens, perform qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood, and calcium along with proper usage of reporting nomenclature with a degree of accuracy equivalent to at least 94%. Given simulated urine specimens, perform tests for physical properties of urine, to include color, appearance, pH, and specific gravity along with proper usage of reporting nomenclature with a degree of accuracy equivalent to at least 90%. Given urine specimens, detect and identify structures commonly seen in normal and abnormal urine sediment to include organized sediment, unorganized sediment,	All previous references.

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Subject and LP No					Lesson Objective	References
					miscellaneous structures and artifacts along with proper usage of reporting nomenclature with a degree of accuracy equivalent to at least 80%.	
Written Examination 08-280-814					Answer questions concerning all material presented during this block of instruction.	All previous references.

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4.

SECTION IV - ANNEX F (Cont)

BIBLIOGRAPHY

Kark, R. M., and others. A Primer of Urinalysis. 2d ed. New York: Hoeber Division of Harper and Row, Publishers, Inc., 1963.

Lippman, Richard W. Urine and the Urinary Sediment: A Practical Manual and Atlas. 2d ed. Springfield, Illinois: Charles C. Thomas, Publisher, 1967.

SUPPLEMENTAL BIBLIOGRAPHY

Davidsohn, Israel and Henry, R. J. Todd-Sanford's Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W. B. Saunders Company, 1967.

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MODIFICATIONS

Lesson Plans - Annex F of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material was not available at time of publication and its exclusion does not alter the value of remaining material.

TM 8-227-6 follows.

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PART THREE

URINALYSIS

Many technicians demonstrate an attitude of disdain toward the daily routine procedures performed in the urinalysis department. Such an attitude is unfortunate and reflects inadequate appraisal of the bearing which the results of these tests may have on clinical diagnosis and treatment. The importance of this work as a screening procedure and an aid in differential diagnosis cannot be overemphasized. Not only may the analysis of urine demonstrate a pathologic condition present in a part of the excretory system, but it also may serve as an index of he general metabolism of the individual. The kidney is responsible for the elimination of waste products (e.g. urea) and also plays an important role in the regulation of body metabolism, i.e., electrolyte and acid-base balance. The maintenance of the proper concentration of all the constituents in plasma is largely dependent upon proper kidney function.

Most new technicians gain their initial laboratory experience in the urinalysis department. As time passes, some individuals tend to regard the work as unimportant and meaningless, particularly because an unusual finding is rarely encountered. With this impression, a tendency to perform slipshod work may develop. It is imperative that the technician performing urinalysis fully realize the great responsibility which is his. Each specimen must be carefully examined as soon as possible after collection. Each test must be conscientiously performed. Continuous learning of the principles of the tests, their meaning and importance, and the possible sources of error which may be encountered, will help to obtain consistently reliable results and provide the training and experience which mark a well-trained technician.

COLLECTION OF SPECIMENS

A random specimen is satisfactory for nost qualitative tests and may be collected t any time. Because of their day to day onsistency, early morning specimens are nost frequently used for analysis. Because he kidney functions at an increased rate fter eating, the presence of abnormal contituents is best demonstrated in specimens oided two to three hours following a meal. t should be remembered that other factors uch as the type and amount of food conumed and the performance of exercise must e considered when interpreting results. or example, a positive urine sugar may be btained after an exceedingly high carbohyrate meal, and this could be a perfectly ormal finding. On occasion, it is necessary to obtain specimens by catheterization, particularly for bacteriological examination.

Twenty-Four Hour Samples

In order to obtain significant results in most quantitative analyses, a carefully collected 24-hour specimen is required. It is essential that a clean container and the proper preservative be used. When the bottle is not in use, it should be kept in a refrigerator.

The method of collection of a 24-hour specimen is as follows:

1. At a specific time on the first day, the patient voids and this specimen is *discarded.* The exact time of voiding is noted and written on the collection container (e.g. 0730).

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2. All other specimens are carefully collected in the container during the next 24 hours (e.g., 1000, 1410, 1740, 2100, 0600, 0700).
3. Exactly 24 hours after the initial voiding (the specimen which was discarded), the final specimen is collected (0730).

NOTE: Regardless of whether a urine specimen is of the random or 24-hour type, it must be thoroughly mixed prior to removing a portion for analysis.

PRESERVATION OF URINE

There is no substitute for a fresh urine specimen and in all cases the analysis should be accomplished as soon as possible. On occasion, however, it is not possible to analyze the sample immediately (e.g., 24-hour specimens). A delay in analysis leads to a degeneration of the formed elements and decomposition of chemical constituents. An example is the breakdown of urea into ammonia by the action of contaminating microorganisms.

Deterioration may be inhibited by the use of some form of preservation. The preferred method of preservation is refrigeration. It is important that the specimen warm to room temperature before an analysis is performed. A number of chemical preservatives can be utilized with varying degrees of effectiveness. The following are recommended for use in conjunction with refrigeration:

1. Toluene (sufficient quantity added to form a thin layer over the specimen) is a very satisfactory inhibiting agent used for the preservation of most chemical constituents. Anaerobic bacteria which might be present in the sample continue to multiply, however. It is necessary either to remove the toluene before measuring portions of the specimen or to pipette from below the surface. The collection bottle should be rinsed with the toluene prior to collecting the sample.
2. Formalin (about 4 drops/100 ml urine) is the best preservative for formed elements. It interferes with some chemical analyses, and should not be used when sugar concentration is to be determined.

If a chemical preservative has been added to a urine specimen the type and amount must be indicated on the label of the collection bottle.

Chapter 16

MACROSCOPIC ANALYSIS

Macroscopic analysis deals with those procedures or examinations which are accomplished without the aid of a microscope. Included in this category are the measurement of volume, noting of color, appearance, eaction (pH) and specific gravity. Prior to performing microscopic and/or chemical tests on a urine specimen, a macroscopic examination is accomplished. Because of the interrelationship which frequently exists between macroscopic, microscopic, and chemical findings on a specimen, this portion of the urinalysis may yield important diagnostic information.

VOLUME

The total 24-hour volume of urine voided by the normal adult is influenced by food and luid intake, temperature (decreased in hot weather), exercise, and excessive use of diuretics such as coffee. The average total volume is approximately 1500 ml with a esser quantity being excreted by children. An abnormal increase in the total volume of urine excreted is called polyuria. Polyuria s associated with such pathologic conditions s diabetes insipidus, diabetes mellitus, and ertain kidney diseases. A reduction in the total volume of urine excreted is called oliguria and this condition is associated with febrile states, excessive vomiting, shock, etc. Anuria refers to complete cessation of urine excretion.

When the total volume of a urine specimen is to be measured, the smallest graduated cylinder which will hold the entire quantity should be used. When measuring the volume of a specimen, the amount of liquid preservative which has been added is not to be included in the total volume.

COLOR

The color of normal urine results from the resence of various pigments and is described in terms of varying shades of yellow. he actual color depends upon the concentraion of the specimen, i.e., the more dilute he specimen, the lighter the color, while onversely, the more concentrated the specimen, the deeper the color. Normal urine is ither "straw," "yellow," or "amber" in olor. Very dilute specimens may be almost "colorless" and should be reported as such. Urines which are very concentrated e usually "dark amber" in color.

Large numbers of erythrocytes in a urine specimen (hematuria) impart a smoky red to dark brown coloration. Hemoglobin in a specimen (hemoglobinuria) imparts a red to red-brown coloration. Hematuria may be differentiated from hemoglobinuria by microscopic examination.

The color of urine may be affected by the presence of a number of other substances including porphyrins, food pigments, dyes, drugs, etc. It is important that the technician note the exact color observed and indicate any changes which occur on standing. Colors ranging from port wine to black are possible and may be clinically significant.

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APPEARANCE

Fresh urine specimens may be clear, hazy, or cloudy. If allowed to stand, all samples become cloudy as bacterial contaminants multiply. It is evident, therefore, that freshly voided urine must be used. If cloudiness is the result of amorphous urates (acid urine) or phosphates (alkaline urine), no clinical significance is attached to said cloudiness. Urates will dissolve if the acid specimen is allowed to stand in warm water. Phosphates dissolve if acid (e.g., 10 percent acetic acid) is added to the specimen.

Cloudiness which does not disappear upon the addition of acid or the application of heat is probably of a cellular nature but may be due to the presence of mucus. A uniform turbidity may be caused by bacterial contamination, while a heavy sediment generally indicates a large number of epithelial cells and/or leucocytes.

Report the appearance of the specimen as "clear," "hazy," "cloudy," or "opaque."

REACTION (pH)

Normal fresh urine may be neutral, acid, or alkaline. The usual pH is about 6, with a possible range of 4.6 to 8. If urine specimens are allowed to remain at room temperature for extended periods of time, they become alkaline because of the conversion of urea to ammonia by contaminating bacteria. Such factors as diet, drug therapy, fluid intake and pathologic conditions influence the reaction. Knowledge of the pH is of value to the technician in identifying crystals and other structures in the sediment during the microscopic examination.

The reaction of urine is usually determined by the use of indicator paper such as pHydrion or nitrazine. The tip of the paper is dipped into the specimen (or a drop may be placed on the paper) and the resulting color is compared with the standard chart supplied with the item. The pH may also be obtained by mixing several drops of urine with a drop of indicator solution, such as universal indicator.

SPECIFIC GRAVITY

The specific gravity of urine depends upon the amount of solid material in solution and the volume of the specimen. Generally, the greater the volume, the lower the specific gravity, and the lighter the color. The converse may also be true. Considerable variation in the specific gravity of random specimens is seen over a 24-hour period. Early morning samples are usually the most concentrated. The majority of normal random specimens have a specific gravity range of 1.003 to 1.030. In pathologic conditions, the extremes of the range are 1.001 and 1.060. The 24-hour specimen may vary from 1.015 to 1.025.

The equipment required for the determination of specific gravity includes a urinometer and glass cylinder. A new urinometer should always be checked prior to use. It is also necessary to periodically check the instruments which are employed daily. This is accomplished by placing the urinometer in distilled water. The reading should be 1.000 at the temperature specified on the urinometer. If a large discrepancy is noted, it is wise to discard the instrument. If the discrepancy is a small one, a correction factor may be used. For example, if a reading of 1.001 is obtained, 0.001 should be subtracted from the specific gravity value obtained from each urine specimen in order to obtain the true reading.

The procedure for measuring the specific gravity of a urine specimen is as follows:

1. Fill the clean cylinder about three-quarters full with a specimen which has been allowed to cool to room temperature.
2. Place the urinometer in the specimen while holding the top between the thumb and forefinger.
3. Spin the instrument so that it rotates free of the sides of the container. Be certain that the bottom of the urinometer is floating and *does not* touch the bottom of the cylinder.
4. When the urinometer stops spinning, read the specific gravity value from the bottom of the meniscus. If the instrument touches the side of the cylinder, repeat the operation.

If the temperature of a urine specimen varies from that at which the urinometer was calibrated, it is necessary to use a correction factor. For each 3°C. above the calibration temperature, add 0.001 to the specific gravity obtained. For each 3°C. below the calibration temperature, subtract 0.001 from the value obtained.

Occasionally, a specimen is received which is of insufficient volume for a specific gravity determination. In such instances, dilute a portion of the urine with distilled water and measure the specific gravity of the dilute mixture. To obtain the approximate reading for the undiluted urine, multiply the numbers after the decimal point by the total volume of urine and water, and divide by the volume of urine diluted. To this figure, add one (1). For example, 20 ml of urine are diluted with 30 ml of distilled water. The specific gravity of this diluted mixture is found to be 1.006. The specific gravity of the undiluted urine is:

$$\frac{.006\times 50}{20}+1=1.015$$

Chapter 17

MICROSCOPIC EXAMINATION

The importance of properly performing microscopic examinations of urinary sediments cannot be overemphasized. Improper preparation of the specimen and faulty use of the microscope are the primary sources of error. In addition, technicians may not possess adequate knowledge of the type and appearance of structures present in the sediment to accurately identify some of the objects observed.

The urine specimen must be fresh since the nature of the sediment changes with time. It is essential that the microscopic examination be performed within a few hours after collection in order to obtain valid results. Refrigeration and or formalization is required if a delay in the analysis is deemed necessary.

PROCEDURE FOR ROUTINE MICROSCOPIC EXAMINATION

Mix the specimen thoroughly, place about 15 ml in a centrifuge tube, and centrifuge at 1,500 rpm for 5 minutes. Without disturbing the sediment, invert the tube, and discard the supernatant fluid. Place the tube in an upright position and allow the drops adhering to the sides to run back into the sediment. Mix the sediment in the remaining fluid by flicking the tube with the finger or stirring with a small glass rod. Using a capillary pipette transfer *one drop* of the mixed sediment to a clean glass slide and cover with a coverslip. Although it is not essential to coverslip the preparation, routine covering facilitates the examination of a uniform layer and protects against contamination of the objectives. It is important that each urine specimen be processed in a similar manner, particularly when daily examinations are performed on the same patient for purposes of comparison.

Scan the entire drop using low power and *moderate* light. When an object is seen which cannot be clearly identified, switch to high power. Report the average number of red cells, white cells, and epithelial cells per high power field (HPF), e.g., occasional rbc, 0–2 wbc/HPF, 15–23 rbc HPF, wbc TNTC (too numerous to count). Using *dim* light, scan the entire drop again for the presence of casts. Casts are reported in terms of the number per low-power field. Report crystals, mucus threads, etc., as occasional, frequent, or many.

Two common sources of error in a microscopic examination are drying of the sediment and placing drops of sediment from too many patients on the same slide. It is recommended that sediments from not more than two patients be placed on the same slide.

ORGANIZED SEDIMENTS

The term organized sediments refers to body cells or their derivatives. Such structures may be found in small numbers in all urine specimens, but if present in any appreciable amount, they are usually associated with a pathological condition. Catheterization of females may be required to distinguish an abnormal increase in the number

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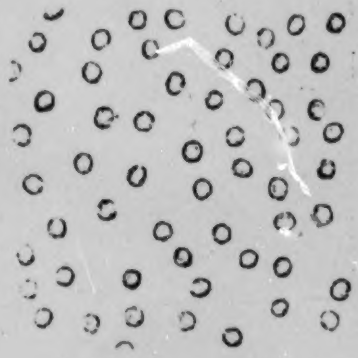
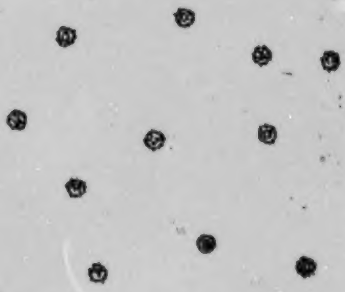
of erythrocytes, leucocytes, and epithelial cells from an increase due to menstrual contamination.

Erythrocytes

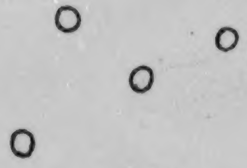
The presence of large numbers of erythrocytes is always pathological when contamination with menstrual discharge may be excluded. Their appearance varies considerably depending upon the reaction, specific gravity, age, etc., of the specimen.



Intact Red Blood Cells. Red cells may be morphologically *intact* and have the characteristic shiny surface with a blue-green tint. When blood is present in a large amount, it will change the color of the urine to a hazy reddish or brown color, (commonly called "smoky").



Crenated Red Blood Cells. In concentrated urine the red cells are often *crenated* with margins displaying numerous sharp angles. This is due to the effect of osmotic pressure removing the internal red blood cell fluid, thus collapsing the cell.



"Ghost" Red Blood Cells. In dilute urine specimens the swollen *ghost* form of the erythrocyte is frequently encountered. The swelling is caused by fluid flowing into the cell as a result of altered osmotic pressure.

In alkaline urine, red blood cells are small in size or may be entirely absent. To differentiate between erythrocytes and leucocytes, yeast cells, or contaminants, add a drop of 10-percent acetic acid to the sediment. Red cells, if present, will dissolve while other structures remain unaffected. If large numbers of red blood cells are present, a positive protein will be obtained from the supernatant fluid of a centrifuged specimen.

Leucocytes

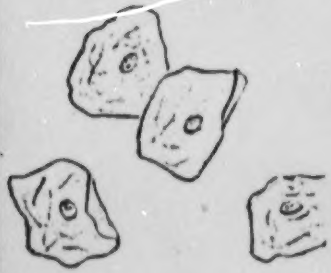
Small numbers of white blood cells are normally present in urine. Any marked increase is indicative of a pathologic condition at some location within the genitourinary tract. Catheterization or a "two-glass test" may be required to distinguish urethral infection from infection of other parts of the genitourinary system. The leucocytes have the same appearance as those observed in a white cell count. If the specimen has an alkaline reaction, the leucocytes may be swollen. The addition of a drop of 10-percent acetic acid not only allows differentiation from erythrocytes but also brings the nuclei more clearly into view. The majority of white cells found are neutrophils.

Leucocytes in Urine. When leucocytes appear in normal or slightly elevated numbers they are reported as number of white cells per high powered field. If present in definitely increased numbers, they are often referred to as *pus cells.*

A positive albumin will usually be obained on the supernatant fluid of a centriuged specimen if large numbers of leuocytes are present.

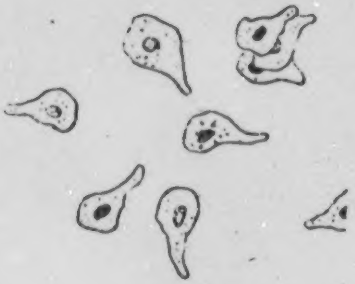
pithelial Cells

Small numbers of the various types of pithelial cells may normally be observed in the sediment. Since distortion from their original appearance usually has occurred, it is generally not possible to accurately state the point of origin of the cells on the basis of their shape. It is, nevertheless, important to report the types of epithelial cells observed.



Squamous Epithelial Cells. The most common type of epithelial cell found in urine is the *squamous* variety. These are large flat cells with small distinct round nuclei. They are sloughed from the ureters, bladder or genitalia (except in specimens from females) and are usually present in relatively small numbers. Specimens from females are commonly contaminated with cells of this type originating from the vagina.

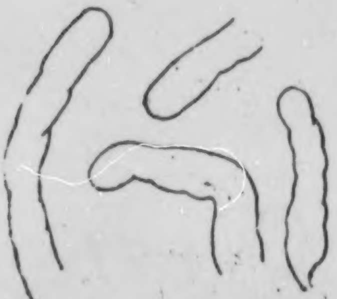
Renal Epithelial Cells. These cells are believed to have their origin in the kidneys. They are slightly larger than leucocytes with a single round nucleus.



Caudate Epithelial Cells. These cells are about three times the size of white cells and are found in many shapes, most of them having tail-like projections. Their origin appears to be the pelvis of the kidney and the neck of the bladder.

Casts

Casts are infrequently found in the urinary sediment of normal individuals. An increase in their number is seen in various types of renal disease and their presence is, therefore, of great importance. Casts are formed in the renal tubules and vary considerably in shape and size according to their origin. Since casts are forced out of the tubules they vary in length, thickness and are often broken at one end. Some casts are readily identified using low power while others require high power for differentiation from other structures. Subdued light must be used when identifying any cast as they are highly refractive. A positive protein is often found when large numbers are present. Casts will dissolve in an alkaline urine, thus it is imperative that urines be analyzed as soon as possible.



Hyaline Casts. The basic substance of casts is a coagulated albuminous material (often referred to as a "hyaline matrix"). Casts consisting exclusively of a hyaline matrix are colorless, slightly refractive, homogeneous, almost completely transparent, and are called *hyaline casts.*

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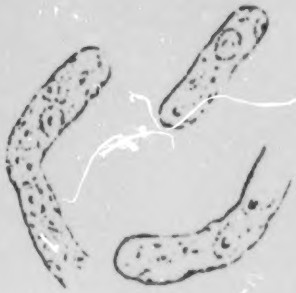
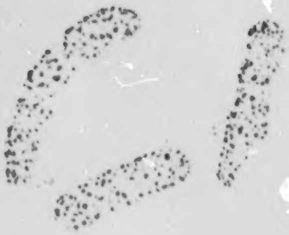
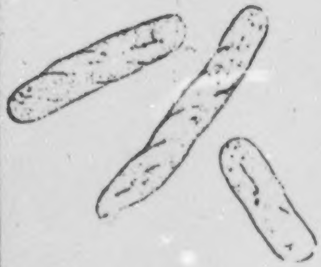


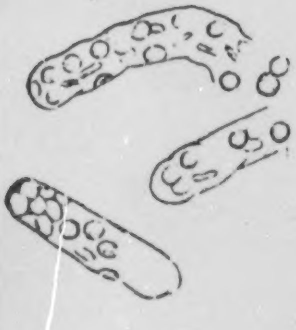
Epithelial Casts. When epithelial cells are sloughed off from the tubules and adhere to, or become incorporated in, hyaline casts, the structure is called an *epithelial cast.* These casts are usually swollen and tinged with a yellow or brown color. They are rarely observed. Ten percent acetic acid brings out the nuclei more distinctly and aids in recognition of the cells.



Coarse Granular Casts. In the event that the epithelial cells do not become immediately incorporated with a hyaline cast, they tend to degenerate into coarse granules. When such coarse granules adhere to the casts, they form *coarse granular casts.*



Fine Granular Casts. These casts are a further degenerated form of epithelial cells in which the granules are smaller in size than the coarse type. The differentiation between coarse and fine granulation is not too important. Clinically, it may be of significance to recognize the difference between a granular cast and a hyaline cast.



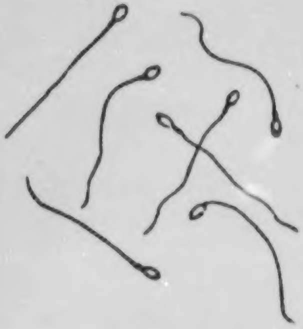
Red Blood Cell Casts. These casts are hyaline casts containing erythrocytes and are usually red or orange in color. The red blood cells contained may be degenerated making identification of the cast difficult.

Cylindroids. Cylindroids resemble casts to an even greater degree than mucus threads, but usually have one tapered end. They have no known clinical importance other than being present in increased numbers in inflammatory conditions.

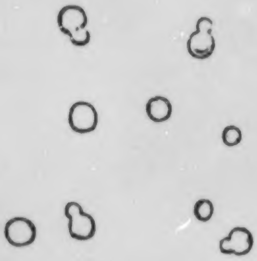
Bacteria, Yeasts, Parasites and Sperm Cells

Bacteria are not present in normal urine except as contaminants. Bacteria multiply rapidly and cause a uniform cloudiness throughout the sample. If found in a freshly voided specimen, urinary tract infection may be indicated. Large numbers may give a false positive test for protein.

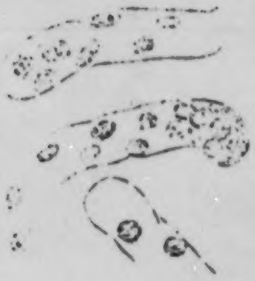
Parasites are sometimes found in urine. Among those which may be observed are *Trichomonas hominis*, *Trichomonas vaginalis* (most common), *Schistosoma haematobium*, and *Enterobius vermicularis*.



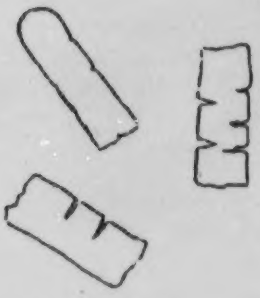
Spermatozoa. These are easily identified by their characteristic shape and affinity for stains (methylene blue or Gram stain). A *verbal* report of the presence of sperm cells in a urine specimen from female patients should be given to the pathologist, laboratory officer, or physician.



Yeast Cells. These cells resemble erythrocytes and leucocytes but usually show characteristic budding. They are nonnucleated and are insoluble in acetic acid. Yeast may be found in the sediment of the urine of a diabetic but generally appear in these specimens as contaminants. Their presence should be reported along with some indication as to numbers present.



White Blood Cell Casts. These casts are hyaline casts containing leucocytes. Finding an occasional white blood cell in casts has no special significance. It is only when the cast is nearly or completely filled with white blood cells that they are reported as white blood cell casts.

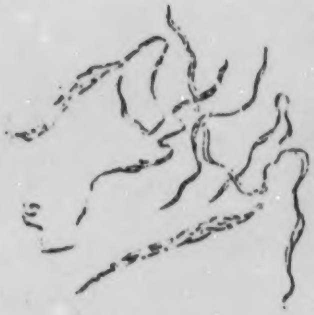


Fatty Casts. When the breakdown of the epithelial lining of the tubules leads to the production of fat droplets instead of granules, *fatty casts* may be formed. The fat droplets are larger than the granules seen in granular casts, and are highly refractile. Fatty casts are insoluble in acetic acid, in contrast to the previously mentioned casts, but are soluble in ether. They stain orange with Sudan III or black with osmic acid, and are rarely observed.

Composite casts are combinations of the various types of casts. Of all the casts this is the one that will be most frequently observed.

Pseudocasts

Occasionally, the inexperienced technician will identify a structure as a cast only to find on reexamination that the object observed resembled a cast but was actually something else.



Mucus Threads. These threads are long, slender, transparent strands which may normally be found in small numbers. Increased numbers tend to be present in various urinary tract infections or irritations. They are often twisted into various formations and this characteristic aids in distinguishing them from casts.

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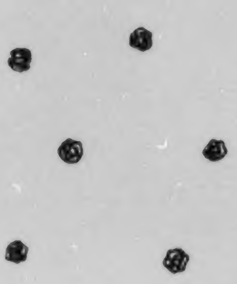
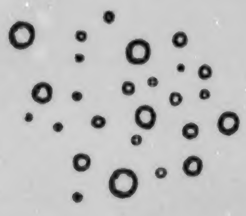
Contaminants Resembling Ogranized Sediments

The main sources of contamination are improperly cleaned specimen bottles and slides. A number of contaminants resemble blood cells and parasites and may be mistaken for these structures.

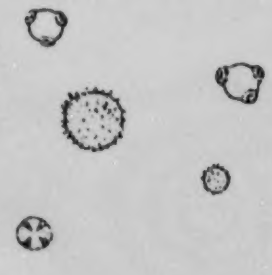
Scratched slides, dirty eyepieces, and smudged objectives often cause confusion. It is wise to rotate the eyepiece periodically to be certain that extraneous structures which may adhere to the eyepiece are not being identified as objects of significance contained in the specimen.



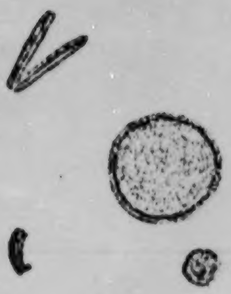
Starch Granules. These granules vary in shape and size. They turn blue-black upon the addition of iodine.



Oil Droplets. Droplets are spherical and show concentric rings of light refraction upon focusing up and down with the fine adjustment. There is a wide variation in size.



Pollen Granules. These granules may be confused with erythrocytes or parasites. They vary in size and appearance according to their source. Those illustrated here represent only a few of the many different types.



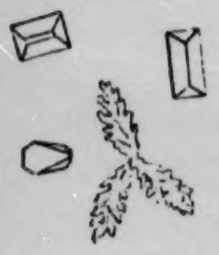
Diatoms. These are one-celled plants which may be introduced into collecting bottles with tap water. Those illustrated here represent only a few of the many different types.

CRYSTALLINE AND AMORPHOUS SEDIMENTS

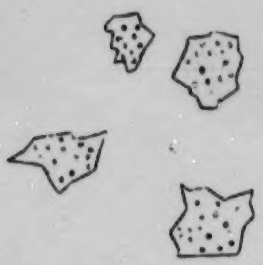
The majority of the crystals which are
und in fresh urine are not clinically signif-
ant. If present in greatly increased num-
rs, however, they may be important. In
ldition, certain pathologic conditions are
companied by the excretion of abnormal
ystals in the urine. An approximate clas-
fication of the unorganized sediments based
on the pH of the sample may be used, but
is division is not absolute. Some crystals
und normally in acid urine may, therefore,
present in a sample which is neutral or
ghtly alkaline. Crystals usually observed
alkaline urine may be present in neutral
ecimens. Upon aging, the number of
ystals appearing in the specimen increases.
It is important that the technician be-
mes thoroughly familiar with the appear-
ce of the crystals most frequently ob-
ved, although they are of little significance unless present in large numbers. The actual number present in the specimen is not noted but they are instead reported as occasional, frequent, or many. Any unusual crystals should be thoroughly examined and every effort made to properly identify them.

"Normal" Crystal Content of Alkaline Urine

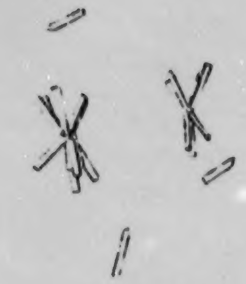
Triple phosphate (ammonium magnesium phosphate), calcium phosphate, amorphous phosphates and ammonium urate crystals are frequently found in alkaline specimens. The phosphates are all soluble in acetic acid and may be differentiated from other crystals by this characteristic. They have no clinical significance unless present in large numbers and are always found in urine which has been standing for an extended period of time.



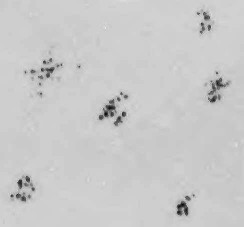
Triple Phosphates. These crystals may appear in neutral or slightly acid, as well as alkaline urine and are almost always rectangular. They usually assume the shape of a coffin lid, but occasionally are found in a feathery or leaflike form.



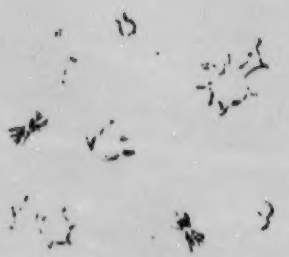
Calcium Phosphate. Calcium phosphate crystals are normally seen as plates with irregular granulation and are found only in alkaline urine.



Dicalcium Phosphate. Dicalcium phosphate crystals may appear in alkaline, neutral, or slightly acid urine in the form of stars or rosettes.



Amorphous Phosphates. The amorphous phosphates are common in alkaline urine and appear as a granular precipitate.



Ammonium Urates. These crystals are precipitated only when free ammonia is present (bacterial action on longstanding specimens), and are, therefore, frequently seen when phosphates are present. They may be found in several different forms, for example, "thorn-apple" crystals, sheaves of fine needles, and dumbbells. Ammonium urate crystals possess a characteristic yellow color.

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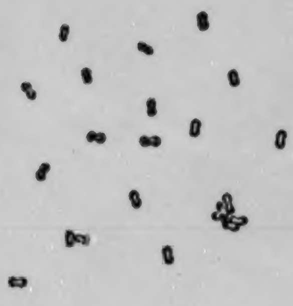
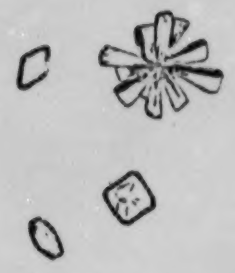


Calcium Carbonate. These crystals occur as amorphous granules or colorless ("dumbbell") spheres. If acetic acid is added to alkaline urine containing calcium carbonate crystals, gas will be evolved as indicated by effervescence.

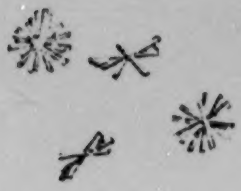
"Normal" Crystal Content of Acid Urine

Uric acid, amorphous urate, and calcium oxalate crystals are often found in acid specimens, particularly after standing for extended periods of time. If uric acid crystals are present in fresh samples the possibility exists that a stone is present somewhere in the renal system. Uric acid and its derivatives will dissolve if the specimen is warmed.

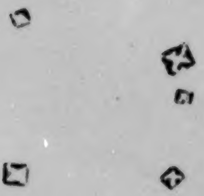


Uric Acid. These crystals are found in many different forms. They are typically yellow or reddish-brown in color and may, like urates, give a cloudy or milky appearance to the specimen.

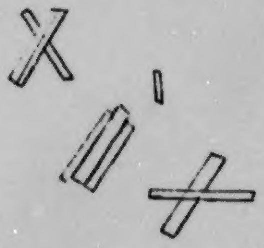
Amorphous Urates. The crystals are common and appear as a granular precipitate. Differentiation between this type of urates and amorphous phosphates may be made by noting the pH of the urine, or the effect of 10-percent acetic acid or gentle heating of the urine.



Sodium Urate. These crystals are usually fan-shaped and may be yellow in color.



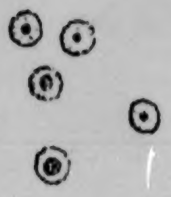
Calcium Oxalate. These crystals are commonly found in acid urine but may be seen in neutral or slightly alkaline specimens. They are rarely of importance and their presence is usually the result of a diet rich in oxalic acid (e.g., tomatoes, spinach, rhubarb and asparagus). Calcium oxalate crystals vary greatly in size and shape but are generally seen as colorless, octahedral (box-shaped) crystals resembling small squares crossed by two intersecting diagonal lines ("envelope" appearance). They may also appear as dumbbells or spheres.



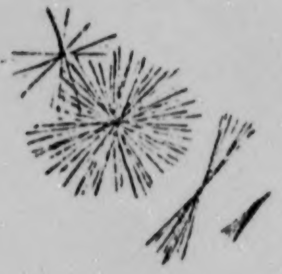
Calcium Sulfate. These crystals, which are rarely observed, are colorless and assume the form of long needles or elongated prisms.

Abnormal Crystals

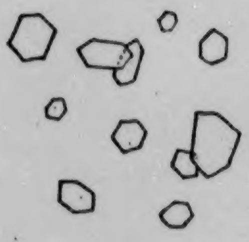
Although small amounts of the chemicals from which "abnormal" crystals are derived are normally present in urine, the appearance of the substance in the crystalline form is frequently of importance.



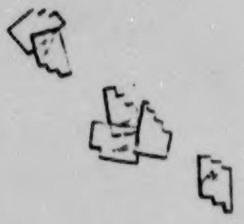
Leucine. Leucine and tyrosine crystals are cleavage products of protein and usually occur simultaneously. It is believed that they are formed as a result of serious liver damage. Leucine crystals are yellowish, oily spheres often possessing radial and concentric striations.



Tyrosine. Tyrosine crystals appear as very fine needles usually arranged in sheaves with a constriction in the middle Both leucine and tyrosine are found in acid specimens.



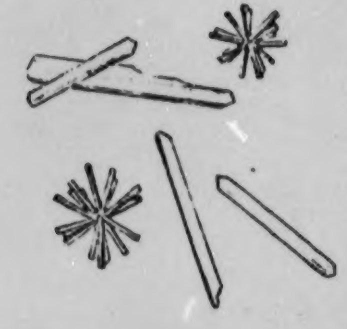
Cystine. Cystine is a breakdown product of protein and appears very rarely. The crystals occur in acid urine as colorless, highly refractile hexagonal plates with well defined edges. In cystinuria an inborn metabolic error, the crystals occur very frequently.



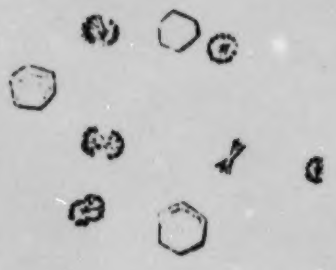
Cholesterol. The cholesterol crystal is another form rarely found. They have a characteristic "missing corner" and appear in acid specimens as large, flat, colorless plates.

Sulfonamide Crystals

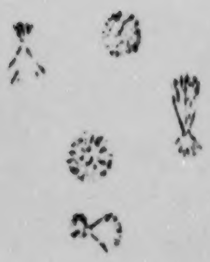
Following sulfonamide therapy, crystals of the drug, or a derivative, may be found in acid specimens. The sulfa compounds are more soluble in an alkaline pH and the maintenance of an alkaline urine during drug administration may be required. The purpose of this alkalinity is to prevent crystallization of sulfa compounds in the kidney tubules with resulting damage. The various sulfa drugs have different crystalline forms which are often colored. A relatively simple way of identifying sulfa crystals is to dissolve the drug being administered in an alkaline solution, evaporate the solution almost to dryness, and compare the resulting crystals with those observed in the urine.



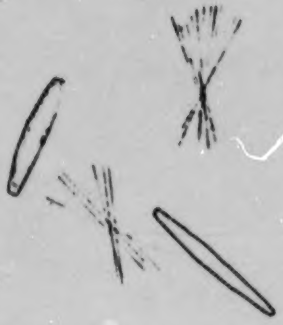
Sulfanilamide. These crystals are seen in the form of long transparent bars or needles which may be grouped in sheaves.



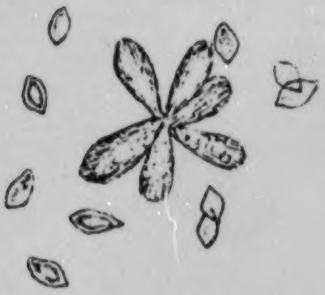
Sulfathiazole. The form assumed by this crystal is either a hexagonal plate or "shock of wheat" with central binding.



Sulfadiazine. These crystals are in the form of "shocks of wheat" with the binding toward one end, or they may resemble burrs.



Sulfaguanidine. These crystals appear as either needles or plates.



Sulfapyridine. Crystals of this drug resemble arrowheads or flower petals when found in urine.

ADDIS SEDIMENT COUNT

The Addis sediment count is used to determine the number of cells and casts excreted during a 12-hour period, after fluid restriction. It is used as an index of the type and degree of kidney disease. Unless the specimen is properly collected and the test carefully performed, it is of no value. It is best to perform two separate Addis counts on successive days in order to obtain any degree of accuracy.

Collection of the Specimen

Instruct the patient to have a normal breakfast including fluids, but to abstain from *all liquids* from this time until the following morning. In addition to having no fluids, advise the individual to avoid excessive quantities of fruit to prevent alkalinization of the urine. Otherwise no change in the diet is necessary. At about noon, the patient should urinate but should not void again until the test is begun. Because of the fluid restriction, this should create no problem. At some suitable hour in the evening, usually about 2000 hours, the patient voids *completely* and the urine is discarded. The exact hour of this voiding is written on the label of the container used to collect the test specimen. If at all possible, the patient should not void again until *exactly 12 hours* have elapsed, at which time the bladder is completely emptied into the collection container. Should it be necessary to urinate prior to the conclusion of the time period, the specimen is collected in the container. Exactly 12 hours after beginning the test, the remainder of the urine is passed into the same container and the time of the final collection is written on the label. If two successive Addis counts are to be performed, the patient is allowed to eat a normal breakfast, with liquids, but thereafter the neces-

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Chapter 18

QUALITATIVE TESTS

In recent years a number of advances have been made in the development of qualitative urinalysis tests. There are available many commercial preparations which have been devised specifically for the rapid detection of certain chemical substances in urine. This improvement has taken the form of increased production of commercial tablets, papers, "stix," etc., designed for a particular analysis. Examples of substances detected using these commercial products are as follows:

Glucose	**Bilirubin**
Protein	**Blood**
Acetone	**Hemoglobin**
Diacetic Acid	**Phenylpyruvic acid**

The tests are based on chemical principles similar to the more lengthy classical tests routinely performed in the laboratory. These preparations have the advantages of saving time and space. Some of the substances that have been common causes of false positive reactions have been eliminated. An example of this is the replacement of the classical copper reduction test for glucose detection with one based upon an enzymatic reaction. The rapid tests are not without limitations, however. Frequently, stability is sacrificed for specificity and sensitivity; and, as a result, false negative reactions may become more common as the commercial preparation ages or deteriorates. Because of the large number of such preparations, no attempt will be made to discuss individual products in this chapter. Routine use of tablets or papers manufactured by reputable firms is recommended for screening procedures. It is essential, however, that the technicians follow the manufacturers' instructions when performing a test.

Contamination from spilled specimens and carelessness are constant possible sources of error. In addition, deterioration of the testing product may occur. It is recommended that confirmatory tests and controls be used, particularly when a new product becomes available or a new technician is being trained in urinalysis. The tendency to use a "sloppy" technique is widespread in qualitative analyses. A valid result is only possible if the outlined technique is explicitly followed. Positive and negative controls should be periodically set up to be certain that the reagents in use are still satisfactory and that the technique being used is correct. If the end point is a white precipitate, the tube should be placed in front of a dark background when the results are interpreted. A colored end point should be observed against a white background. Any doubtful results should be repeated.

Entries showing the results of examinations performed should be made on appropriate laboratory forms. They should be neat, clearly legible, and complete. Negative findings should be reported by recording the entire word, not by symbol.

ACETONE AND DIACETIC ACID

(Rothera)

Principle (Ref: 25, 52)

Sodium nitroprusside reacts with acetone and diacetic acid, in a buffered alkaline medium, to produce a red to purple color.

REAGENTS

1. Ammonium sulfate crystals
2. Ammonium hydroxide, concentrated
3. Sodium nitroprusside reagent (209)

TECHNIQUE

1. Place about 5 ml of urine in a test tube.
2. Add ammonium sulfate crystals with agitation until the solution is saturated.
3. Add 5 drops of concentrated ammonium hydroxide and mix.
4. Add 5 drops of sodium nitroprusside solution and mix.
5. Allow to stand for 15 minutes.
6. A red to purple color which does not fade is positive.
7. A red to purple color which fades during the first 5 minutes is negative.
8. A brown or orange color is negative.

DISCUSSION

1. The test is not valid if the patient has received PSP or BSP dye.
2. The test is more sensitive for diacetic acid than acetone.
3. Beta-hydroxybutyric acid does not give a positive test.
4. Prepare the nitroprusside solution just before use.
5. Lower concentrations of acetone and/or diacetic acid show more of a red color while higher concentrations produce a distinct purple color.

BENCE–JONES PROTEIN

Principle (Ref: 36)

Bence-Jones protein is differentiated from other proteins by its insolubility at 40–60°C. and its solubility above 60°C.

REAGENT

Acetic acid, 10 percent (2)

TECHNIQUE

1. Perform the analysis on a fresh specimen.
2. If the urine is cloudy, filter.
3. Perform the sulfosalicylic acid test for protein. If a negative result is obtained, Bence-Jones protein is *not* present.
4. If a positive sulfosalicylic acid test is obtained, boil a portion of the urine, agitating continuously, and add several drops of 10 percent acetic acid. *Filter while hot.* Separation of Bence-Jones from other proteins is accomplished in this step.
5. Allow the filtrate to cool.
6. Place 5 ml of the filtrate in a test tube. Place the tube in a water bath and heat gently. Take frequent temperature readings.
7. If Bence-Jones protein is present, the urine will develop a turbidity at about 40°C. and precipitation will occur between 40°C. and 60°C.
8. Continue to heat to boiling. A precipitate due to Bence-Jones protein will partially or totally disappear.
9. Allow to cool. If Bence-Jones protein is present, the precipitate will reappear between 40°C. and 60°C.

DISCUSSION

1. Bence-Jones protein may precipitate at room temperature if the specimen is very acid. Use a fresh sample.
2. Avoid overacidification with acetic acid.
3. Careful control of the temperature is imperative.
4. A "false positive" test may be obtained if mucin is present. All positive results should be checked by performing a mucin analysis.
5. Bence-Jones protein is an abnormal substance often found in the urine of individuals with multiple myeloma. It may also be present in leukemia and other diseases.

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BILE PIGMENT

Diazo Method

Principle (Ref: 41, 52)

Bile pigments are removed from the specimen by absorption with barium sulfate which is formed from the reaction between barium chloride and the sulfate present in the urine. The van den Bergh test, in which Ehrlich's diazo reagent and bile pigment react to form a pink-red color, is performed on the washed precipitate of barium sulfate.

REAGENTS

1. Barium chloride, 10 percent (33)
2. Sulfanilic acid, 0.1 percent (231)
3. Sodium nitrite, 0.5 percent (207)
4. Diazo reagent (66)
5. Ethyl alcohol, 95 percent
6. Sodium phosphate, 3.6 percent (211)

TECHNIQUE

1. Place about 10 ml of urine in a centrifuge tube.
2. Add 5 ml of 10 percent barium chloride solution, mix, and centrifuge.
3. Decant the supernate, add about 5 ml of water, mix and centrifuge.
4. Decant the supernate.
5. Add 0.5 ml of diazo reagent to the precipitate and mix.
6. Add 2 ml of 95 percent ethyl alcohol and mix.
7. Add 0.5 ml of sodium phosphate solution and mix.
8. If bile pigments are present, a pink-red color will appear.

DISCUSSION

1. The test is not valid if the patient has received an injection of PSP or BSP dye.
2. This analysis is very sensitive provided the sodium nitrite and diazo reagent are freshly prepared.
3. Normally, bile pigments do not occur in the urine in detectable amounts. The determination may be used as a liver-function test in conjunction with other analyses.

Fouchet's Method

Principle

Bile pigments are adsorbed from the specimen by a precipitate of barium sulfate which results from the reaction of barium chloride and the sulfate present in the urine. After washing, the precipitate is treated with a ferric chloride in trichloroacetic acid solution. If bile pigments are present, a green to blue colored derivative is formed.

REAGENTS

1. Barium chloride, 10 percent
2. Fouchet's reagent

TECHNIQUE

1. Place about 10 ml of urine in a centrifuge tube.
2. Add 5 ml of 10 percent barium chloride solution, mix, and centrifuge.
3. Decant the supernate, add about 5 ml of water, mix and filter.
4. Place the filter paper on a piece of dry paper.
5. Add 2 drops of Fouchet's reagent to the precipitate.
6. If bile pigments are present, a green to blue color will appear.

DISCUSSION

1. This test is sensitive and may be used instead of the diazo method.
2. This test, like the diazo procedure, may be employed as a method for evaluation of liver function.

BENZIDINE TEST FOR BLOOD

Principle (Ref: 36, 45)

Hemoglobin liberates oxygen from hydrogen peroxide. The oxygen released oxidizes benzidine to produce a green to blue color. A positive control is prepared to check the sensitivity and stability of the reagents.

REAGENTS

1. Benzidine solution (37)
2. Hydrogen peroxide, 3 percent (103)

TECHNIQUE

1. Place 2 ml of urine in a test tube.
2. In a second tube, add 1 drop of blood to 2 ml of the urine (positive control).
3. Add 2 ml of benzidine solution to each tube and mix.
4. Add 1 ml of hydrogen peroxide solution to each tube and mix.
5. A green to blue color is positive for blood.

DISCUSSION

1. The greatest source of error is unsatisfactory reagents.
2. A "false negative" may result if the specimen is too dilute. The test should be performed on the sediment of a centrifuged sample if the specific gravity is below 1.015.
3. Formalin will produce a false positive test.

CALCIUM

(Sulkowitch)

Principle (Ref: 36, 45)

Calcium reacts with ammonium oxalate to form insoluble calcium oxalate. The degree of precipitation is roughly proportional to the amount of calcium in the sample. The additional chemicals present in the Sulkowitch reagent prevent precipitation of other urinary substances.

REAGENT

Sulkowitch reagent (247)

TECHNIQUE

1. Place 5 ml of urine in a test tube. If the specimen is not acid in reaction, acidify with 10 percent acetic acid.
2. Add 5 ml of Sulkowitch reagent, mix, and allow to stand for 5 minutes.
3. Absence of turbidity is reported as "negative" and indicates a lack of calcium in the specimen.
4. A slightly turbid suspension is reported as "decreased."
5. A "normal" report is given when a definite turbidity is obtained, through which newspaper print may be read.
6. An opaque turbidity or a heavy flocculent milky turbidity is reported as "increased."

DISCUSSION

1. This test is a useful rapid screening procedure.
2. Analysis of a 24-hour specimen will provide more information than testing a random specimen.
3. The patient should be on a low calcium diet for 72 hours prior to collection of the specimen to prevent undue dietary influence on the examination.
4. High calcium excretion may result from hyperparathyroidism. Tetany may be accompanied by a very low calcium output and, in some instances, a complete absence may be evident.

DIACETIC ACID

(Gerhardt)

Principle (Ref: 36, 45, 52)

Ferric ions react with diacetic acid to give a red-wine color. Diacetic acid, when heated in the presence of a dilute acid, decomposes and renders a negative test.

REAGENTS

1. Ferric chloride, 10 percent (78)
2. Nitric acid, concentrated

TECHNIQUE

1. Place 5 ml of urine in a clean test tube.

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2. Add ferric chloride dropwise until the phosphates are precipitated.
3. If a precipitate develops, filter.
4. To the urine filtrate, add 2 ml of the ferric chloride.
5. A red-wine color indicates the presence of diacetic acid or one of several interfering substances.
6. To 5 ml of urine add 5 ml of distilled water and one drop of concentrated nitric acid. Reduce the volume of solution to 5 ml by boiling rapidly. Allow to cool and filter, if necessary. Repeat test as above.
7. The absence of a red-wine color is a positive test for diacetic acid.

DISCUSSION

1. In the first test the development of a red-wine color indicates the presence of diacetic acid or some interfering drug requiring a confirmatory test.
2. The absence of a red-wine color in the confirmatory test indicates that the color in the first test was due to diacetic acid which was either boiled off or oxidized to acetone.
3. A red-wine color is produced by phenols, coal tar, antipyretics, salicylates, sodium bicarbonate, and other compounds.
4. This test is less sensitive to diacetic acid than the Rothera test (i.e., more diacetic acid is present when a positive test is obtained using the Gerhardt procedure). Results obtained by the Gerhardt procedure may thus give the physician an indication of the severity of the condition of ketonuria.

HEMOSIDERIN

(Rous)

Principle (Ref: 45)

Unstained hemosiderin granules are brown in color. When reacted with potassium ferrocyanide and hydrochloric acid, the iron in the pigment is converted to Prussian blue.

REAGENTS

1. Potassium ferrocyanide, 2 percent (160)
2. Hydrochloric acid, 3 percent (101)

TECHNIQUE

1. Centrifuge about 15 ml of fresh urine and decant the supernate.
2. Examine part of the sediment microscopically for the presence of brown granules, particularly within epithelial cells.
3. To the remainder of the sediment, add 2 ml of potassium ferrocyanide solution and 2 ml of hydrochloric acid and mix.
4. Allow to stand for 10 minutes, centrifuge, and decant the supernate.
5. Examine the sediment microscopically for the presence of blue granules.

DISCUSSION

1. Hemosiderin is a pigment, containing iron, which is *not* normally found in urine. It may be present in cases of hemochromatosis and hemolytic anemia.

HOMOGENTISIC ACID

(Ferric Chloride Test)

Principle (Ref: 31, 52)

Ferric chloride reacts with the phosphates in urine to form insoluble ferric phosphate. If homogentisic acid is present, a green to blue color will appear.

REAGENT

Ferric chloride, 10 percent. (78)

TECHNIQUE

1. Place 5 ml of urine in a test tube.

2. Add ferric chloride solution drop by drop.
3. The appearance of an unstable green to blue color is positive for homogentisic acid.

DISCUSSION

1. Only a few drops of ferric chloride are required.
2. The color is not stable.
3. Homogentisic acid reduces Benedict's reagent with the eventual appearance of a yellow precipitate. This property may be used as a confirmatory test.
4. Upon standing, urine containing homogentisic acid darkens.
5. The primary reason for performing the homogentisic acid test is to differentiate between melanogenuria and alkaptonuria. The ferric chloride test is also used to test for the presence of melanogen.
6. Alkaptonuria refers to the excretion of alkapton bodies, of which homogentisic acid is the primary compound. It is associated with abnormal amino acid metabolism and may be of a hereditary nature.
7. Refer to the determination of melanin (melanogen).

INDICAN

(Obermayer)

Principle (Ref: 36, 45)

Bile pigments, which interfere with the test, are removed by adsorption with barium chloride. The filtrate is reacted with Obermayer's reagent (ferric chloride in hydrochloric acid) which oxidizes the indican. A chloroform extraction is performed and the extract is examined for the presence of indican which is indicated by a blue or, rarely, a red color. Sodium thiosulfate is added to remove any color resulting from the presence of iodides or thymol.

REAGENTS

1. Barium chloride, 10 percent (33)
2. Obermayer's reagent (133)
3. Chloroform
4. Sodium thiosulfate crystals

TECHNIQUE

1. Place 10 ml of urine in a test tube, add 2 ml of 10-percent barium chloride, mix, and filter.
2. In a second tube, treat 10 ml of a *normal* control urine in the same way.
3. Transfer 5 ml of the filtrates to test tubes, add 5 ml of Obermayer's reagent, and mix.
4. Add 2 ml of chloroform and mix by inversion.
5. Allow to settle.
6. If the chloroform (lower) layer is colorless, the tube is negative.
7. If a blue or red color is observed, transfer the chloroform layer to another test tube, add a small crystal of sodium thiosulfate, and mix.
8. If the blue or red color disappears, the test is negative.
9. If the color intensity of the patient's tube and normal control are identical, the test is negative.
10. If the color of the patient's tube is more intense than that of the normal control, report as positive.

DISCUSSION

1. The intensity of the color is proportional to the concentration of indican.
2. A red color is rarely observed.
3. Formalin may give a false negative result.
4. Normally, indican is present in small amounts in urine. For this reason, a normal control, for comparison purposes, is necessary.
5. Large quantities are found in such pathologic conditions as intestinal obstruction or excessive putrefaction in the intestines. The urine may become green-blue on standing.

MELANOGEN
(Ferric Chloride Test)

Principle (Ref: 31, 52)

Ferric chloride reacts with the phosphates in urine to form insoluble ferric phosphate. If melanogen is present, it adheres to the ferric phosphate and produces a brown or black colored precipitate.

REAGENT

Ferric chloride, 10 percent (78)

TECHNIQUE

1. Place 5 ml of urine in a test tube.
2. Add ferric chloride solution drop by drop.
3. The appearance of a gray color followed by a brown to black precipitate is positive for melanogen.

DISCUSSION

1. Only a few drops of ferric chloride are required. The precipitate will dissolve in an excess.
2. Melanogen does not reduce Benedict's reagent. A green-black precipitate may form if a great deal of melanogen is present but the brown-yellow precipitate obtained with homogentisic acid does not appear.
3. Upon standing, urine containing melanogen slowly darkens. This, by itself, is not diagnostic.
4. Melanogen is the colorless chromogen of melanin. It is found in the urine of individuals with melanotic tumors.
5. Refer to the determination of homogentisic acid.

MUCIN

rinciple (Ref: 45)

Mucin is precipitated, without heating, by cetic acid.

EAGENT

Glacial acetic acid

ECHNIQUE

1. Add 6 ml of water to 2 ml of filtered urine and mix.
2. Add glacial acetic acid drop by drop until the specimen becomes very acid in reaction.
3. If the specimen becomes turbid upon addition of the acetic acid, the test is positive for mucin.

DISCUSSION

1. The test is useful for differentiation of mucin from serum protein or Bence-Jones protein, since the latter compounds do not precipitate.
2. Mucin in high concentration will give a false positive test for sugars.
3. The exact nature of mucin is not known. It is present in increased amounts in renal inflammation.

PORPHOBILINOGEN

inciple (Ref: 36, 52)

Bile pigments are removed by adsorption ith barium chloride. Ehrlich's reaction etween porphobilinogen and or urobilinoen and paradimethylaminobenzaldehyde in ydrochloric acid is performed. Differentiation between urobilinogen and porphobilinogen is then carried out on the basis of chloroform and water solubility properties.

REAGENTS

1. Barium chloride, 10 percent (33)
2. Ehrlich's reagent (72)

3. Sodium acetate, saturated solution (183)
4. Chloroform

TECHNIQUE

1. Add 2 ml of 10-percent barium chloride to 10 ml of *fresh* urine in a test tube, mix, and filter.
2. Place the filtrate in a large test tube, or separatory funnel. Add 10 ml of Ehrlich's reagent and mix
3. Add 10 ml of saturated sodium acetate and mix.
4. Add 5 ml of chloroform and mix.
5. If neither the aqueous nor chloroform (lower) layer is red, the test is negative for both urobilinogen and porphobilinogen.
6. If the aqueous layer is red and chloroform layer colorless, the test is positive for porphobilinogen.
7. If the chloroform layer is red and the aqueous layer colorless, the test is positive for urobilinogen.
8. If both layers are red, extract with chloroform until the chloroform layer is colorless. If the aqueous layer is still red, the test is positive for both porphobilinogen and urobilinogen.

DISCUSSION

1. Use a fresh, acid urine specimen.
2. Refer to the determination of porphyrins for further information.
3. Porphobilinogen may be found in porphyria. Upon standing, it is converted to uroporphyrin.

PORPHYRINS

Principle (Ref: 31, 36, 52)

The presence of excess porphyrins is indicated by characteristic color changes upon treatment with chemicals, fluorescence in ultraviolet light, and noting specific absorption bands obtained in spectroscopic analysis.

REAGENTS

1. Sulfuric acid, 10 percent (245)
2. Acetic acid, glacial
3. Ether (75)
4. Hydrochloric acid, concentrated

SPECIMEN COLLECTION AND PRESERVATION

1. A 24-hour specimen collected in a brown bottle, using toluene as a preservative, is most satisfactory.
2. Place a portion of the specimen in sunlight for several hours. The development of a port-wine color may indicate the presence of porphyrins.

CHEMICAL TECHNIQUE

1. Place 50 ml of urine in a beaker and add 50 ml of 10-percent sulfuric acid.
2. Boil for 1 minute.
3. Set aside for 12 hours.
4. A clear, lemon to red-brown solution is negative.
5. A red-brown solution with a marked purple-red granular precipitate is positive.

FLUORESCENCE TECHNIQUE

1. Place 25 ml of urine in a separatory funnel, add 10 ml of glacial acetic acid, and mix.
2. Add 50 ml of ether and shake for one minute, removing the stopper periodically.
3. Allow the layers to separate.
4. Draw off the bottom (aqueous) layer into a second separatory funnel and extract once more with 50 ml of ether.
5. Combine the ether extracts in one of the separatory funnels.
6. Examine the aqueous fraction under ultraviolet light. Red fluorescence indicates a positive test for uroporphyrin.
7. Examine the ether fraction under ultraviolet light. Red fluorescence indicates a positive test for coproporphyrin.

SPECTROSCOPIC TECHNIQUE

1. Examine a portion of urine using a

spectroscope. Alkaline porphyrin absorption bands are seen in the orange, yellow, green, and green-blue portions of the spectrum.

2. Add 1 ml of concentrated hydrochloric acid to 5 ml of urine and mix. Examine with a spectroscope. Acid porphyrin absorption bands are seen in the yellow, yellow-green, and orange-yellow portions of the spectrum.

DISCUSSION

1. Normal urine may contain small amounts of porphyrins but the concentration is insufficient to obtain a positive test.
2. A porphobilinogen determination should be run at the same time.
3. If possible, all three techniques should be performed when screening for the presence of porphyrins.
4. In the spectroscopic procedure, the alkaline porphyrin lines appear with untreated urine or after a base has been added to the specimen.
5. Porphyrins may be found in increased amounts in various metabolic diseases and toxic conditions.

PROTEINS

Heat and Acetic Acid Method

Principle (Ref: 36, 45)

Proteins are coagulated by heat. Dilute acetic acid is added to dissolve precipitated phosphates and enhance coagulation of protein.

REAGENT

Acetic acid, 10 percent (2)

TECHNIQUE

1. Filter the specimen or use the supernatant obtained after centrifugation for the microscopic examination.
2. If the urine is alkaline or neutral, add 10-percent acetic acid drop by drop until it is *just* acid.
3. Place about 10 ml of the clear urine in a test tube.
4. Heat the upper portion of the tube until the contents boil, for several seconds.
5. Add 2 drops of 10-percent acetic acid, then heat to boiling once again.
6. The formation of a persistant white precipitate is positive for protein.
7. Report as:
    negative—urine remains clear
    trace—faintly visible turbidity when observed against dark back-background (less than 10 mg per 100 ml)
    1+ —definite turbidity (10–50 mg per 100 ml)
    2+ —heavy turbidity but no floccules (50–200 mg per 100 ml)
    3+ —heavy cloud with flocculation (200–500 mg per 100 ml)
    4+ —heavy cloud with heavy flocculation to a solid precipitate (above 500 mg per 100 ml. Solid at 3 *gm* per 100 ml).

DISCUSSION

1. If the urine is not initially acid, the later addition of acetic acid may be insufficient to obtain heat coagulation of protein.
2. If too much acid is added, traces of protein may be missed.
3. Mucin may give a false positive test. Add 2 ml of saturated sodium chloride solution to the urine prior to boiling to avoid mucin effect. Alternatively, perform a mucin test, filter, and test the filtrate.
4. Normal specimens may contain a small amount of protein but this is generally not detectable.
5. Albumin is the usual serum protein found in proteinuria, but globulins may be present.
6. Proteinuria may occur in a number of diseases and the importance of a properly performed test cannot be overemphasized.

Sulfosalicylic Acid Method

Principle

Proteins are coagulated by a dilute sulfosalicylic acid solution.

REAGENT

Sulfosalicylic acid, 3 percent (232)

TECHNIQUE

1. Filter the specimen or use the supernate obtained after centrifugation for the microscopic examination.
2. If the urine is alkaline or neutral, add 10-percent acetic acid drop by drop until it is *just* acid.
3. Place about 2 ml of the clear urine in a test tube.
4. Add 2 ml of sulfosalicylic acid and mix.
5. The formation of a white precipitate is positive for protein.
6. Report as:
    - negative—urine remains clear
    - trace—faintly visible turbidity when observed against dark background (less than 10 mg per 100 ml)
    - 1+ —definite turbidity (10–50 mg per 100 ml)
    - 2+ —heavy turbidity but no floccules (50–200 mg per 100 ml)
    - 3+ —heavy cloud with flocculation (200–500 mg per 100 ml)
    - 4+ —heavy cloud with heavy flocculation (above 500 mg per 100 ml)
7. If the specimen cannot be cleared by centrifugation, dilute 2 ml of urine with 2 ml of water and compare with the sulfosalicylic acid-urine mixture. A positive protein reaction will show a distinct difference.

DISCUSSION

1. Mucin may give a false positive test.
2. Bence-Jones protein may give a positive test.
3. High concentrations of uric acid may give a false positive test.
4. This test is very sensitive and its routine use is recommended.

SUGAR

(Benedict)

Principle (Ref: 36, 45)

Cupric sulfate in alkaline solution is reduced to cuprous oxide by heating with glucose and other reducing agents. The degree of reduction is indicated by the presence of a yellow to red precipitate.

REAGENT

Benedict's qualitative reagent (35)

TECHNIQUE

1. Add 8 drops (0.4 ml) of urine to 5 ml of Benedict's qualitative reagent in a test tube and mix.
2. Place in a boiling water bath for 5 minutes.
3. Allow to cool.
4. Report as: (See figure 1.)
    - negative—no change or green color *without* precipitate.
    - 1+ —green-yellow precipitate (0.5–1.0 gm per 100 ml)
    - 2+ —Olive green precipitate (1.0–1.5 gm per 100 ml)
    - 3+ —yellow precipitate (1.5–2.5 gm per 100 ml)
    - 4+ —orange to brick red (2.5–4.0 gm per 100 ml or higher)

DISCUSSION

1. If the urine is positive for protein, heat to boiling, add 2 drops of 10-percent acetic acid and filter. Perform the Benedict test on the cool filtrate.
2. The amounts of reagent and specimen used are important.

Figure 1. Interpretation of Benedict's Qualitative Sugar Test.

3. Formalin and chloroform give false positive tests.
4. A white or grey precipitate results from the presence of phosphates.
5. Do *not* use Benedict's quantitative reagent to perform this test.
6. A positive test only indicates the presence of an increased concentration of reducing substances as this test is not specific for glucose or other sugars.
7. The small amount of reducing agents normally present in urine is insufficient to give a positive result.
8. If a positive test is obtained, perform an acetone determination.
9. Diabetes mellitus and various dietary habits may result in glucosuria.

IDENTIFICATION OF SUGARS BY PAPER CHROMATOGRAPHY

Principle (Ref: 52)

Sugars are separated by using the principles of paper chromatography. Indentification is accomplished by means of prepared standards.

REAGENTS

1. Solvent mixture (224)
2. Sugar standards (230)
3. Color-developing solution (61)

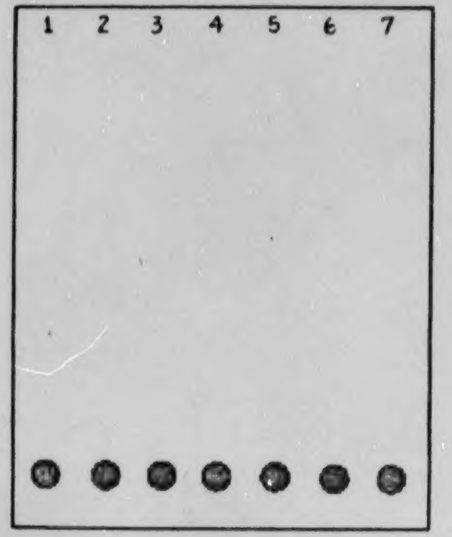
PREPARATION OF CHAMBER

1. A large glass container with a solid flat coverplate is required.
2. Clean the chamber and plate thoroughly.
3. Place solvent mixture in the chamber to a depth of about one-half inch. Cover and seal with stopcock grease.
4. Allow to stand for 24 hours. The atmosphere will become saturated.
5. Clean the chamber carefully after use.

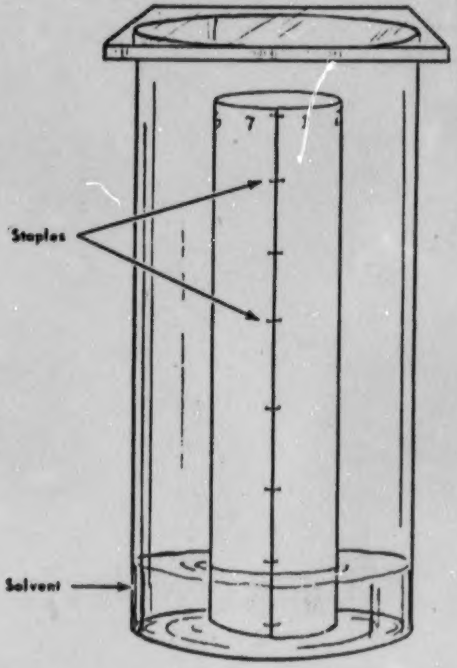
TECHNIQUE

1. Place a piece of Whatman 1 filter paper on a clean surface. The size used will vary according to the dimensions of the chamber.
2. Draw a line about 10 cm from the bottom of the sheet.
3. Apply about 0.05 ml of the urine on one small area of the line. The urine should be applied a little at a time, and allowed to dry between additions. It is best to apply the fluid in a small circular spot.
4. At spaced intervals along the line, apply 0.01 ml of each of the standards.
5. Allow all spots to dry.
6. Roll the paper into a cylinder, overlapping the edges slightly and staple together. The line with the spots should be on the outside and none of the spots should be placed near the staples (see fig. 1).

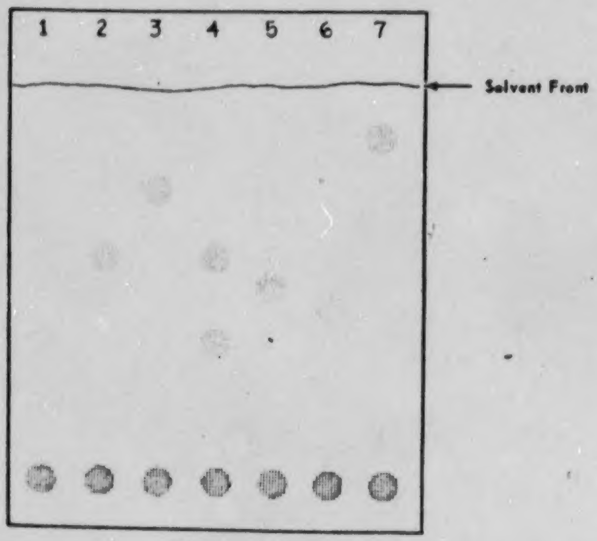
7. Place the paper in the chamber and quickly replace the glass plate.
8. Allow the fluid to flow until the solvent front has traveled at least 30 cm, then remove from the chamber.
9. Remove the staples and allow the paper to air dry.
10. Saturate the paper with dinitrosalicylic acid solution by spraying with an atomizer. Avoid an excess.



Chromatogram with Standards and Unknowns



Chromatography Tank With Chromatogram



Chromatogram After Staining

LEGEND

1. Lactose standard
2. Glucose standard
3. Fructose standard
4. Unknown urine (contains glucose and lactose)
5. Unknown urine (contains galactose)
6. Galactose standard
7. Pentose standard

Figure 2. Chromatographic Apparatus for Sugar Analysis.

11. Allow to air dry, then place in a 100°C. oven for 10 minutes.
12. The standards will appear as brown spots which have migrated various distances from the line of application.
13. Compare brown spots appearing above the original point of application of the urine sample with the standard spots.

DISCUSSION

1. Set up the chromatography apparatus on a solid surface away from direct sunlight and heat.
2. Avoid the fumes from the solvent and color-developing solutions.
3. On a separate sheet of paper, note the initial locations of the urine and standard spots.
4. The spots should be as small as possible. The use of a hair dryer between additions of small volumes is recommended.
5. The cylinder should be large enough to stand without support.
6. If a yellow-orange spot appears above the area where the urine is applied, it is probably creatinine.
7. Differentiation of sugars in a urine specimen has always been a problem in the laboratory. The use of chemical tests and the phenylhydrazine reaction is more difficult and time-consuming than the use of Chromatography.

SULFONAMIDES

(Hallay)

Principle (Ref: 36)

Sulfonamide compounds react with crude cellulose, in the presence of acids, to form a yellow to orange color.

REAGENT

Hydrochloric acid, 25 percent (97)

TECHNIQUE

1. Place 2 drops of urine on a blank strip of newspaper or a paper towel.
2. Add 1 drop of 25-percent hydrochloric acid.
3. The immediate appearance of a yellow to orange color is positive for a sulfonamide compound.

DISCUSSION

1. Do not use filter or writing paper.
2. The test is used as a screening procedure to determine if the patient is on sulfonamide therapy or if crystals observed in microscopic examination are sulfonamide in nature.

UROBILIN

(Schlesinger)

Principle (Ref: 36, 45, 52)

Bile pigments are removed by adsorption with barium chloride. After conversion of urobilinogen to urobilin by the addition of iodine, alcoholic zinc acetate is added which produces a green fluorescence due to the formation of a urobilin-zinc compound.

REAGENTS

1. Barium chloride, 10 percent (33)
2. Lugol's solution (120)
3. Zinc acetate, saturated alcoholic solution (269)

TECHNIQUE

1. Add 3 ml of 10-percent barium chloride to 15 ml of urine, mix and filter.
2. Place 10 ml of the filtrate in a test tube, add 4 drops of Lugol's solution, and mix.
3. Add 10 ml of saturated zinc acetate solution, mix and filter.
4. Hold the tube against a dark back-

ground and concentrate light upon it with a lens.

5. A green fluorescence is positive for urobilin.

DISCUSSION

1. A small amount of urobilin is normally present in urine, but this quantity is not detectable by the test. A positive result is, therefore, indicative of increased urobilin concentration.
2. Urobilin is the colored pigment derived from urobilinogen. It imparts an orange tinge to urine.
3. For further information, refer to the urobilinogen determination.

UROBILINOGEN

Principle (Ref: 36, 45, 52)

Urobilinogen reacts with Ehrlich's reagent (paradimethylaminobenzaldehyde in hydrochloric acid) to form a red color. Bile pigments are first removed by adsorption with barium chloride.

REAGENTS

1. Barium chloride, 10 percent (33)
2. Ehrlich's reagent (72)

TECHNIQUE

1. Add 2 ml of 10-percent barium chloride to 10 ml of *fresh* urine in a test tube, mix, and filter.
2. Place 5 ml of the filtrate in a tube, add 1 ml of Ehrlich's reagent, and mix.
3. Allow to stand for 3–5 minutes.
4. Look through the solution from the top against a white background.
5. A red color is positive for urobilinogen.

DISCUSSION

1. A pink or light red color indicates a normal amount of urobilinogen. A cherry-red color is found if an increased concentration of urobilinogen is present.
2. The urine sample must be fresh since urobilinogen will be converted to urobilin upon standing.
3. More information is gained by a semiquantitative or quantitative analysis.
4. Separation of porphobilinogen from urobilinogen is accomplished as outlined under the porphobilinogen determination.
5. Urobilinogen is produced in the intestine by the action of bacteria on bile pigments. Part of it is absorbed and excreted in small amounts in the urine. Increased concentrations are found in conditions associated with excessive red cell breakdown. Obstructive jaundice is accompanied by decreased amounts or complete absence of urobilinogen in the urine.

Note: Misnumbered p. #39 is missing.
However, all material is included.

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ANNEX G

BASIC PROCEDURES IN SEROLOGY

OBJECTIVE: Given known controls and unknown test samples, perform routine serological procedures, to include screening tests for rheumatoid arthritis, heterophil antibodies, pregnancy, and syphilis with an acceptable degree of accuracy.

Subject and LP No	Lesson Objective	References
Introduction to Serology 08-280-821	Define immunology and serology as they apply to the detection of disease. List properties of antigens and antibodies. List the various antigen-antibody reactions as they apply to serology. Define cross-reactivity in a serological reaction. Define zonal reactions as applied to antigen-antibody reactions. List current acceptable methods for collecting, processing and shipping of serological specimens.	TM 8-227-1, chap 1-4, and 15.
Immunological Screening Tests 08-280-824	Describe rheumatoid factor and the disease condition associated with it. Define heterophil antibodies and list those of medical significance with their associated disease conditions.	TM 8-227-1, chap 7 and 11.
Serological Tests for Syphilis 08-280-827	List and briefly discuss the stages of syphilis. Define "non-specific" as the term applies to syphilis testing and list four such tests. Define "specific" as the term applies to syphilis testing and list four such tests.	TM 8-227-1, chap 6.

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Subject and LP No	Lesson Objective	References
Immunological Tests for Pregnancy 08-280-830	List and classify by principle the four types of immunological pregnancy tests.	TM 8-227-1, chap 13.
Serological Techniques 08-280-832	Given serum and urine specimens and commercial test kits, and employing manufacturer's directions and procedure handouts. Perform serological tests for rheumatoid arthritis, mononucleosis, pregnancy, and syphilis with an acceptable degree of accuracy.	All previous references.
Graded Serological Procedures 08-280-833	Given serum and urine specimens and commercial test kits, and employing manufacturer's directions and procedure handouts, accurately perform serological tests for rheumatoid arthritis, mononucleosis, and pregnancy with at least 80% accuracy, and serological tests for syphilis with at least 95% accuracy.	All previous references.
Written Examination and Critique 08-280-835	Answer questions concerning all material presented during this block of instruction.	All previous references.

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SECTION IV - ANNEX G (Cont)

BIBLIOGRAPHY

Hynson, Wescott. *Manufacturer's Instructions Supplied with the Rapid Plasma Reagin Test Kit*, n.d.

Stonier, Peter F. and Lewis, Cherryl. *Workshop Manual in Pregnancy Testing*. Kamloops, British Columbia: Annual Postgraduate Course in Medical Technology, Royal Inland Hospital, n.d.

SUPPLEMENTAL BIBLIOGRAPHY

Carpenter, Phillip L. *Immunology and Serology*. 2d ed. Philadelphia: W. B. Saunders Company, 1965.

Davidsohn, Israel, and Henry, R. J. *Todd-Sanford's Clinical Diagnosis by Laboratory Methods*. 14th ed. Philadelphia: W. B. Saunders Company, 1967.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-821
038

Introduction to Serology (3 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-1, Chaps 1, 2, 3, 4, and Chap 15, pp 15-4 thru 15-6.

STUDY ASSIGNMENT: After class study TM 8-227-1, Chap 15, pp 15-4 thru 15-6

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B2H, 92B3H, or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard and eraser; PA system w/lapel microphone; M 08-280-821-1, -2, issued at start of class.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture (3 hours)

I. INTRODUCTION (5 min)

A. Opening Statement: The importance of serology as an aid in the diagnosis of bacterial diseases has been recognized since the late 19th century. However, from about 1910 until the late 1950's, the rate of progress in this field did not keep pace with earlier developments. Interest in the subject was revived primarily by development of new discoveries in the fields of virology and tissue compatibility, as well as by an increasing venereal disease rate. For these reasons the laboratory specialist must have a thorough knowledge of the entities and basic principles involved in serological testing.

B. Objectives

1. Define immunology and serology as they apply to disease.

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*This lesson plan supersedes LP 08-280-821, 057

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2. List the properties of antigens and antibodies.
3. List the various antigen-antibody and complement-dependent reactions.
4. Define cross-reactivity.
5. Define zonal reactions and methods of problem resolution.
6. List the methods of preparation of specimens prior to performance of serological tests.
7. List current acceptable methods for collection, processing and shipping of serological specimens.

C. Class Procedure and Lesson Tie-in

II. EXPLANATION (105 min)

A. Definitions

NOTE: Refer to M 08-280-821-2

1. Immunology
2. Serology

QUESTION: What is a definition of serology?

ANSWER: A branch of immunology that deals primarily with antigen-antibody reactions and the detection of antibodies in the patient's serum.

3. Immunity
4. Antigen
5. Antibody
6. Complement

B. Properties of Antigens and Antibodies

1. Antigens

   a. Usually protein

- b. Molecular weight generally high (10,000 to 100,000)
- c. Foreign to body
- d. Stimulate antibody response
- e. Parts -- carrier and haptene
- f. Specificity -- haptene

NOTE: Illustrate carrier and haptene on chalkboard using a circle for the carrier and a pyramid for the haptene.

2. Antibodies
   - a. Molecular weight generally very high (150,000 to 800,000)
   - b. Specificity -- receptor site
   - c. Produced primarily in the reticuloendothelial system
   - d. Gamma globulin
   - e. Visibility of reaction

NOTE: Illustrate on the chalkboard a circle with a section cut out to fit the pyramid-shaped haptene drawn previously.

QUESTION: What are some of the properties of antigens and antibodies?

ANSWER: (Antigens) -- generally protein in nature; high molecular weight; foreign to the body; stimulate definite antibody response.

(Antibodies) -- gamma globulin in nature; produced in the reticuloendothelial system; very high molecular weight; show specific response to antigens.

C. Complement
   1. Group of 11 serum proteins
   2. Heat-labile
   3. Bacteriolytic and cellulolytic properties

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4. Interferes with some serological tests by causing lysis of cells

5. Inactivate at 56° C for 30 minutes

SECOND HOUR

D. Types of Antigen-Antibody Reactions

   1. Agglutination

      a. Cellular or particulate antigen and specific antibody

      b. Macroscopic reaction

   2. Flocculation

      a. Particulate antigen and soluble antibody

      b. Microscopic reaction (microscopic agglutination)

      c. Routinely performed in serological laboratories

   3. Lysis

      a. Cellular antigen and specific antibody

      b. Cells destroyed or dissolved (*e.g.*, hemolysis)

   4. Precipitation

      a. Soluble antigen and soluble antibody

      b. Forms insoluble, measurable complex.

   5. Neutralization

      a. Antigen's toxic effect nullified

      b. Toxin-antitoxin reactions

E. Cross-Reactivity

   1. Causes false positive reactions

   2. Single organisms may contain many antigenic substances

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NOTE: Show cross-reactivity through use of chalk drawings of one organism (carrier) with several haptenes (varying shapes) and several antibodies with similiar-shaped receptor sites.

QUESTION: What is cross-reactivity?

ANSWER: The ability of closely related antibodies to react with the same antigen.

F. Zonal Reactions

1. Cause false negative reactions

2. Prozone

   a. Antibody in excess of antigen

   b. Patient has very active disease

   c. Detect or correct by diluting specimen (antibody)

QUESTION: What is a prozone reaction?

ANSWER: A test-system situation whereby the antibody level (titer) is in excess of the antigen level (titer) and the anticipated reaction does not take place because of the excess antibody.

3. Postzone

   a. Antigen in excess of antibody

   b. Often due to technician error

   c. Carefully repeat the test

THIRD HOUR

G. Specimen Handling

1. Collection

   a. Venipuncture, capillary puncture

   b. Spinal tap

   c. Urine by clean catch

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2. Processing

   Centrifuge and aspirate supernatant into labelled, clean test tube

3. Preservation

   a. Serum inactivation contraindicated

   b. Methods

      (1) Chemical - add merthiolate - preserves indefinitely

      (2) Freezing - preserves indefinitely at minus 20° C

      (3) Refrigeration - preserves for 72 hours at 4-6° C

4. Shipping

   a. Use proper-sized, approved containers

   b. Seal individual specimens with tape or paraffin

   c. Pack with padding to guard against breakage

5. References

   a. Laboratory SOP - good but often incomplete

   b. Reference Laboratory Shipping Manual -- best, most complete

H. Questions From Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Definitions

2. Properties of Antigens and Antibodies

3. Complement

4. Types of Antigen-Antibody Reactions

5. Cross-Reactivity
6. Zonal Reactions
7. Specimen Handling

B. Closing Statement: Immunity may be considered the body's most potent defense against invading pathogenic organisms. Because of this phenomenon, serology, or the detection of antibodies which may destroy the antigenic organisms or nullify their effects, is one of the most important phases of medical technology.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1

Primary hours	3
Secondary hours	0
TOTAL	3

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-821-1
LABORATORY SCIENCES DIVISION 117
CLINICAL MICROSCOPY BRANCH

Serology Objectives

A. INTRODUCTION

1. Define immunology and serology as they apply to disease.
2. List the properties of antigens and antibodies.
3. List the various antigen-antibody and complement dependent reactions.
4. Define cross-reactivity.
5. Define zonal reactions and methods of problem resolution.
6. List the methods of preparation fo specimens prior to performance of serological tests.
7. List current acceptable methods for collection, processing and shipping of serological specimens.

B. IMMUNOLOGICAL SCREENING TESTS

1. Describe the rheumatoid factor and the disease condition associated with it.
2. Define heterophil antibodies and their association with disease conditions.
3. Identify the principles of the common immunological screening tests as given in class.

C. SEROLOGICAL TESTS FOR SYPHILIS

1. List and briefly discuss the stages of syphilis.
2. Define "specific tests" as the term applies to syphilis testing. List two such tests.
3. Define "nonspecific tests" as the term applies to syphilis testing. List four such tests.
4. State the principles of the nonspecific tests used in serological testing for syphilis.
5. Select the procedural steps for specimen preparation for serological tests for syphilis.

D. IMMUNOLOGICAL TESTS FOR PREGNANCY

1. Name the types of urine specimens commonly collected for serological study. Specify reasons for their collection.
2. List and classify by principle the four types of immunological pregnancy tests.

E. SEROLOGICAL TECHNIQUES

1. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for rheumatoid arthritis.

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2. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for mononucleosis.
3. Given urine specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for pregnancy.
4. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for syphilis.

F. GRADED SEROLOGICAL PROCEDURES

1. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for rheumatoid arthritis to within at least 80% accuracy.
2. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for mononucleosis to within at least 80% accuracy.
3. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for syphilis to within at least 95% accuracy.
4. Given urine specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for pregnancy to within 80% accuracy.

SFC Fredric B. Fudge 52

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-824
LABORATORY SCIENCES DIVISION 057
CLINICAL MICROSCOPY BRANCH

Immunological Screening Tests (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom and Laboratory

REFERENCES:

TM 8-227-1, chap 7 and 11.
Ackerman, P. G., and others. Bray's Clinical Laboratory Methods. 7th ed. Saint Louis: C. V. Mosby Company, 1968, pp 188 thru 191, p 519.
Davidsohn, Israel, and Henry, John Bernard. Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W. B. Saunders Company, 1969, pp 280 thrue 292.
Manufacturers' Test Literature:
RA Test: Hyland Laboratories, Los Angeles, California.
Mono-Test: Wampole Laboratories, Stamford, Connecticut.

STUDY ASSIGNMENT: After class read TM 8-227-1, chap 7 and 11.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-824-1, M 08-280-824-2, and M 08-280-824-3, issued in class (one each per student)

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; PA system with lapel mike; RA, and Mononucleosis test kits (one each per two students); known controls and unknown test samples.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: Modern medicine is a fast-moving science with many great advances rapidly following one another. In keeping with this fast pace, many of the classic, time consuming

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*This lesson plan supersedes LP 08-280-824, 103.

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tests, once the latest available, have been modified, refined, and improved to the point where results are now obtainable in a matter of minutes. In your role as military medical laboratory specialists, you will encounter a number of these rapid screening tests. A knowledge of the principles and mechanics of each is essential if your are to perform these procedures accurately.

B. Objectives

1. Describe rheumatoid factor and the disease condition associated with it.

2. Define heterophil antibodies and list those of medical significance and their associated disease conditions.

C. Class Procedure and Lesson Tie-in

Lecture to be followed by a demonstration, practical exercise, and a graded practical examination.

II. EXPLANATION (30 min)

A. Rheumatoid Factor (RF)

1. Definition

a. Macroglobulin (antibody-like)

b. Reacts with normal human gamma globulin

2. Medical significance

a. Present in 80 to 90 percent of rheumatoid arthritis patients

b. Essential to diagnosis of rheumatoid arthritis

3. Detection

a. Found in serum (most common specimen)

b. Found in synovial fluid (sometimes here first)

QUESTION: What type of antibody is the rheumatoid factor?

ANSWER: A macroglobulin.

QUESTION: Where may RF be detected?

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ANSWER: In serum or synovial fluid.

B. Heterophil Antibodies

1. Definition

    a. Closely related to one another

    b. Show affinity for several different antigens

    c. All agglutinate sheep red blood cells

2. Medical significance

    a. Forssman (F)

        (1) Naturally occurring

        (2) Generally low titer

    b. Serum sickness (SS)

        (1) Present in allergic reactions due to horse serum

        (2) Treated symptomatically

    c. Infectious mononucleosis (IM)

        (1) Known as "great imitator"

        (2) Thought to be viral in nature

        (3) Results in lost man-hours (4 to 8 week recovery period)

        (4) Primary age group: 16 to 25 years of age

        (5) Spread by droplet dispersion

        (6) Symptoms many and varied

QUESTION: What one property of heterophil antibodies enables us to test for their presence?

ANSWER: They all agglutinate sheep red blood cells.

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QUESTION: List the three types of heterophil antibodies.

ANSWER: Forssman, serum sickness, and infectious mononucleosis.

QUESTION: Of the three heterophil antibodies, which is most important?

ANSWER: Infectious mononucleosis.

III. SUMMARY (5 min)

A. Review of Main Points

1. Rheumatoid Factor

2. Heterophil antibodies

B. Closing Statement: During this block of instruction we have seen that there are common diseases for which screening tests are readily available in medical laboratories. These tests are easily performed and are a great aid to the physician, not only in diagnosing disease but in ruling out disease conditions. The accurate performance of these screening tests is essential to proper laboratory medicine.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1

Primary hours	1
Secondary hours	0
TOTAL	1

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-827
038

Serological Tests for Syphilis (3 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: Review all previous lecture material and mimeos. TM 8-227-1, Chapter 6.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; overhead projector; PA system with lapel mike.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (10 min)

A. Opening Statement: Syphilis is one of the oldest recorded diseases in history. Because of the increasing number of cases of syphilis each year, it is important as laboratory specialists to fully understand syphilis, its stages, and the serological tests for its detection.

B. Objectives

1. List and briefly discuss the stages of syphilis.
2. Define "specific tests" as the term applies to syphilis testing. List two such tests.
3. Define "nonspecific tests" as the term applies to syphilis testing. List four such tests.
4. State the principles of the nonspecific tests used in serological testing for syphilis.
5. Select the procedural steps for specimen preparation for serological tests for syphilis.

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*This lesson plan supersedes LP 08-280-827, 057

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C. Class Procedure and Lesson Tie-in

Following this lecture there will be four hours of practical exercise on RPR procedure.

II. EXPLANATION (100 min)

A. Causative Agent

1. Treponema pallidum

a. Spirochete

QUESTION: What is a spirochete?

ANSWER: It is a spiraled-shaped bacteria.

b. Light-sensitive

c. Heat-sensitive (35 - 37° C)

d. Oxygen-sensitive

e. Cannot be grown on artificial media

NOTE: There are three other types of Treponematosis; *T. pertenue*, *T. cuniculi*, and *T. carateum*.

B. Type of Syphilis

1. Congenital

a. Mode of transmission - from mother to fetus across placenta.

b. Treatment - usually curable if treated *in utero*.

2. Acquired syphilis

a. Primary stage

(1) Symptoms

(a) Chancre

(b) Enlargement of area lymph glands

(2) Serological test results

(a) Usually negative

(b) Darkfield microscopy positive

b. Secondary stage

(1) Symptoms

(a) Localized or generalized rash

(b) Chancre may or may not be present

(2) Serological test results

(a) Earliest stage for positive

(b) Usually positive

c. Latent Phase

(1) Symptoms

(2) Serological test results

d. Tertiary Stage

(1) Symptoms

(a) Central nervous system (CNS) involvement

(b) Cardiovascular problems

(2) Serological test results

(a) Serum - positive

(b) CSF - positive

C. Serological Tests for Syphilis

1. Specific tests

a. Definition - a test that uses a specific treponemal antigen to detect a specific substance produced as a result of syphilis.

b. Antigen - Treponema pallidum

c. Antibody - anti-treponemal antibody

d. Tests

(1) Treponema pallidum immobilization (TPI)

(2) Fluorescent treponemal antibody (FTA)

2. Nonspecific tests for syphilis

a. Definition - a test that uses a non-treponemal antigen to detect a non-specific substance (reagin) that may be produced by conditions other than syphilis.

b. Antigen - cardiolipin

c. Antibody - reagin

d. Tests

(1) Kline

(2) Mazzine

(3) Wasserman

(4) CLMF - Cardiolipin Microflocculation

(a) Reagent

(1) Cardiolipin - beef heart extract

(2) Lecithin - plant extract

(3) Cholesterol - increase surface tension

(b) Storage

(1) Dark at room temperature

(2) CLMF emulsion - 24 hours

(c) Shelf-life - indefinite

(5) Rapid plasma reagin (RPR)

QUESTION: What are you detecting with this test?

ANSWER: Reagin

(a) Reagent - CLC complex on carbon particles

(b) Choline chloride inactivates complement

(c) Shelf-life - 3 months

D. Questions From Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Causative agent

2. Types of syphilis

3. Serological tests for syphilis

B. Closing Statement: CDC in Atlanta reports that in age group 15-24 one out of every 5 has syphilis. In Texas in 1976 there was a 42% increase. Because of these facts one can see why these tests are key procedures in any laboratory. Therefore, you as laboratory specialists should be thoroughly familiar with them.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1

Primary hours	3
Secondary hours	0
TOTAL	3

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-830
038

Immunological Tests for Pregnancy (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom and Laboratory

REFERENCES:

TM 8-227-1, Chapter 13, Para 13-1-b
Biochemistry Workshop Manual in Pregnancy Testing, Annual Postgraduate Course in Medical Technology, Royal Inland Hospital, Kamloops, B.C.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; PA system with lapel mike; magnetic placards; overhead projector. (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: Although pregnancy is usually determined by clinical evaluation, in certain instances the medical officer desires to confirm this condition before conclusive clinical evidence is present. Recently, a number of immunological pregnancy tests, superior to the older biological procedures, have become available to the laboratory. As medical laboratory specialists, you must not only be able to perform these tests and interpret the results obtained, but also understand the principles involved.

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*This lesson plan supersedes LP 08-280-830, 057

B. Objective

1. Name the types of urine specimens commonly collected for serological study. Specify reasons for their collection.

2. List and classify by principle the four types of pregnancy tests as given in class.

C. Class Procedure and Lesson Tie-in

None

II. EXPLANATION (35 min)

NOTE: Use magnetic placards to emphasize main points throughout lecture (see Annex A).

A. Body Chemistry Involvement in Pregnancy Testing

1. Human chorionic gonadotropin

a. Production

b. Occurrence

c. Level

2. Specimen collection and rationale

a. First morning urine

b. Random urine

c. Serum or plasma

B. Principles of Pregnancy Tests

1. Agglutination inhibition

a. Gravindex^R

b. Latex-HCG

c. Potential sources of error

QUESTION: In vitro tests for pregnancy are based upon the presence of what substance in the urine and serum of pregnant women?

ANSWER: Chorinonic gonadotropic hormone.

2. Hemagglutination inhibition
    a. Pregnosticon^R
    b. Potential sources of error

NOTE: Use chalk drawing to depict positive and negative reactions in the hemagglutination inhibition technique and explain how to read each (see Annex B).

3. Direct agglutination
    a. DAP Test^R
    b. Potential sources of error

QUESTION: What is the indicator used in the Pregnosticon^R test for pregnancy?

ANSWER: Red blood cells.

4. Biological Pregnancy Tests
    a. Various animals used
    b. Urine injected into animal
    c. Reasons for not using biological tests
        (1) Cost
        (2) Time
        (3) Space limitation

III. SUMMARY (5 min)

A. Review of Main Points
    1. Body chemistry involvement in pregnancies
    2. Principles of pregnancy tests

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B. Closing Statement: Immunological tests for pregnancy are accurate and reproducible when performed correctly. These tests are a great aid to the medical officer and may be ordered for a variety of reasons. It is the medical laboratory specialist's responsibility to fully understand and be able to accurately perform these procedures.

INSTRUCTIONAL HOURS

Number of instructors required - - - - - 1

Primary hours	1
Secondary hours	0
Total	1

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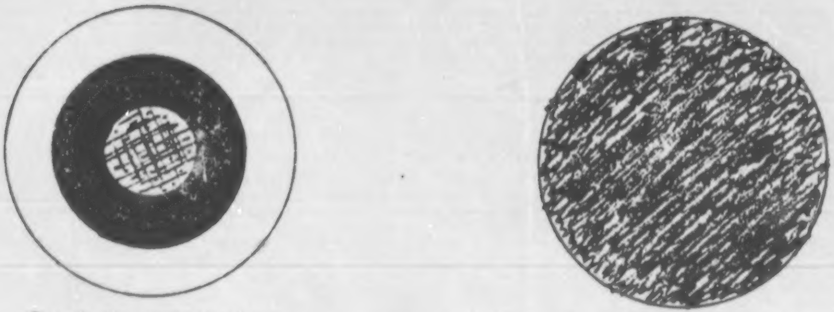
ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

1. BODY CHEMISTRY
2. HUMAN CHORIONIC GONADOTROPIN
3. SPECIMEN COLLECTION
4. TEST PRINCIPLES
5. AGGLUTINATION INHIBITION
6. HEMAGGLUTINATION INHIBITION
7. DIRECT AGGLUTINATION
8. GRAVINDEX TEST
9. PREGNOSTICON TEST

ANNEX B
INSTRUCTIONAL AIDS

Chalk Drawing



Positive reaction

Negative reaction

Hemagglutination Inhibition Test Results

NOTE: This chalk drawing is used in Part IIB2.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-832
038

Serological Techniques (11 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Laboratory

REFERENCES: All previous references

STUDY ASSIGNMENT: Review all previous lecture material and mimeos

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor for 5 students

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; overhead projector; PA system with lapel mike.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: During this exercise you will begin your practical work in the detection of rheumatoid arthritis, mononucleosis, pregnancy, and syphilis. These hours present the basic techniques and give you the practice necessary for you to perform these procedures as a laboratory specialist.

B. Objective

1. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for rheumatoid arthritis.

2. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for mononucleosis.

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*This lesson plan supersedes LP 08-280-832, 067.

3. Given urine specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for pregnancy.

4. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for syphilis.

C. Class Procedure and Lesson Tie-in

Students are permitted to take breaks at appropriate times during the application.

II. APPLICATION (580 min)

A. Instructors' activities

1. Before class prepare unknown samples and known controls according to Annex A.

2. Issue equipment and reagents according to Annex B.

3. Supervise students and correct observed errors on an individual basis.

4. Discuss individual results with student.

5. Allow sufficient time at end of exercise for storage of equipment, reagents, and specimens, and cleaning of work areas.

B. Students' Activities

1. Receive equipment, reagents, and specimens from instructor.

2. Perform procedures using M 08-280-832-3, M 08-280-832-4, M 08-280-832-5, and M 08-280-832-6.

3. Record results and hand to instructor.

4. Discuss problems, if any, with instructor.

5. After completion, store reagents, equipment, and specimens, and clean up work area.

III. SUMMARY (15 min)

A. Questions from Students

B. Review

1. Present observed problems

2. Request students to offer solutions to problems

3. Evaluate students' solutions

C. Closing Statement: These practical exercises have presented the basic techniques for the detection of rheumatoid arthritis, mononucleosis, pregnancy and syphilis.

INSTRUCTIONAL HOURS

Number of instructors required - - - - - - - 1 per 5 students

Primary hours	11
Secondary hours	0
Total	11

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038

ANNEX A
INSTRUCTIONAL AIDS

Preparation of Specimens

A. Rheumatoid Arthritis

1. Prepare sufficient 10 x 75 mm test tubes, numbered consecutively with "R" prefix (i.e., R1, R2, R3) to provide 10 samples per student plus 20 extra. Store labeled tubes in 90 hole test tube racks.

2. Remove, at random, a maximum of five tubes per each 15 tubes in the 90 hole rack; set these aside, recording the tube numbers. These tubes will be used to contain positive samples.

3. Perform a serial dilution on a bottle of RA positive control serum to determine the highest dilution which will yield an acceptable positive result. Make dilutions with glycine saline.

4. Count the number of positive tubes set aside and multiply this total by 2 to determine the total volume (in ml) of positive samples required for student samples. Add 10 ml to this figure to allow for spillage and/or replacement.

5. Pools sufficient bottles of RA positive control serum to yield the total volume (as determined in step 4 above) of the proper dilution (as determined in step 3 above).

6. Dilute the pooled control serum and test to insure that an acceptable positive reaction is obtained. Dilute with glycine saline and add thimerosal as preservative.

7. Prepare sufficient negative specimen to provide 2.0 ml for each negative sample tube. Use normal saline, adding bovine albumin to provide proper surface tension.

8. Use food coloring to adjust colors of all samples until they are visually similar.

9. Dispense proper specimens into proper tubes (2.0 ml/tube) and refrigerate.

10. Set up control serum for students, using the positive and negative specimens.

B. Infectious Mononucleosis

1. Prepare sufficient 10 x 75 mm test tubes, numbered consecutively with "M" prefix (i.e., M1, M2, M3, etc.) to provide 10 sample tubes

per student plus 20 extra. Store labeled tubes in 90 hole test tube rack.

2. Remove, at random, a maximum of 5 tubes per each 15 tubes in the 90 hole rack; set these aside, recording the tube numbers. These will be used to contain positive samples.

3. Pool positive patient serum. (Positive serum for infectious mononucleosis should be obtained through liaison with local medical facilities).

4. Set up a twofold serial dilution on the serum and perform a slide test for mononucleosis on each tube.

5. Select the highest dilution which gives a distinct, readable positive reaction.

6. Dilute the pooled serum to provide 2.0 ml for each tube set aside in step 2 above, using the dilution selected in step 5 above.

7. Prepare sufficient negative specimen to provide 2.0 ml for each negative sample tube (those tubes remaining in the 90 hole racks). Use normal saline, adding bovine albumin to provide proper surface tension.

8. Use food coloring to adjust the colors of all samples until they are visually similar.

9. Retest the positive samples to insure that an acceptable reaction is obtained.

10. Dispense designated specimens into proper tubes (2.0 ml per tube) and refrigerate.

11. Set up control serum for students, using the positive and negative specimens.

C. Pregnancy

1. Using adhesive tape and ball point pen, consecutively number sufficient 10 x 75 mm test tubes to provide 10 tubes per student plus about 30 extra tubes. .

2. Using 90 hole test tube racks, arrange the labeled test tubes in numerical order in groups of 90 tubes.

3. At random, remove a maximum of 5 tubes per each 15 tubes in 90 hole rack; set these aside to use as containers for positive specimens.

4. Record the numbers of the positive specimens to use by the instructor as a key. Example:

Pregnancy Test Key

1-100 1, 2, 7, 14, 18, 21, 26, 33, 39, 47, 53, 58, 62, 69, 74, 75, 77, 83, 85 87, 94, 98, 100

5. Dilute HCG - positive urine samples with normal saline to prepare working urine specimen. These positive samples are to be obtained from local medical facility. The quantity prepared should be sufficient to provide 2 ml for each positive specimen tube. Check working urine specimen for reactivity, using slide test for pregnancy. Use normal saline for negative specimens. Match colors of all specimens with food coloring to prevent color identification of specimens by students. Store specimens in a refrigerator.

6. Dispense 2 ml of the proper specimen into the proper tube set up in step 3 above.

7. Set up control urine for students, using the positive and negative specimens.

D. Syphilis

1. Prepare sufficient 10 x 75 mm test tubes, numbered consecutively with different colored tape to provide 10 samples per student with 20 extra.

2. At random, remove a maximum of 25 tubes from each group of 100. These tubes will be used as reactive specimens. All remaining tubes will be used for nonreactive specimens.

3. Record the numbers of reactive to be used by instructor as a key.
1-100 R: 1, 2, 7, 14, 18, 21, 26, 33, 39, 53, 56, 58, 62, 69, 70, 75, 79, 80, 83, 94, 97, 98, 99, 100

4. Titer the pooled control serum and dilute with normal saline to produce 250 ml of reactive specimen. Using 22% bovine albumin, insure that there is a protein concentration of about 2 gm % in the specimen. (20 ml of 22% bovine albumin in 250 ml of normal saline is sufficient. The protein is required to allow the specimen to spread out to the border of the 18 mm circle. Add albumin to normal saline to produce nonreactive specimens.

5. Check the specimens to insure their reactivity.

6. Dispense 2 ml of the appropriate specimen into each of the tubes set up in steps 2 and 3 above.

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7. Refrigerate all dilutions and specimen tubes until needed.

8. Set up control serum for students, using the reactive and nonreactive specimens.

ANNEX B
INSTRUCTIONAL AIDS

Reagents and Equipment

A. Rheumatoid Arthritis	To each Student
RA Latex Globulin Reagent	1 bottle
Glass Test Slide	2
Dispenstirs (to deliver 0.05 ml)	1 box
Stirrers	1 box
RA Positive control	1 tube
RA Negative control	1 tube
Kimwipes	1 box
B. Infectious Mononucleosis	
Horse RBC Antigen suspension	1 bottle
Mono positive control	1 tube
Mono negative control	1 tube
Glass test slide	2
Stirrers	1 box
Dispenstirs (to deliver 0.05 ml)	1 box
Kimwipes (already issued with RA material)	1 box
C. Pregnancy	
Anti-HCG	1 bottle
Latex particles coated with HCG	1 bottle
Glass test slides	2
Stirrers	1 box
Capillary tubes (to deliver 0.03 ml)	1 box
Bulb	1
Kimwipes (already issued with RA material)	1 box
Pregnancy positive control	1 tube
Pregnancy negative control	1 tube
D. Syphilis (Rapid Plasma Reagin)	
RPR Rotator	1
RPR 18 mm circle	1 box
RPR Antigen	1 bottle
20 gauge needle without bevel	1
Dispenstirs (to deliver 0.05 ml)	1 box
Kimwipes (already issued with RA material)	1 box
RPR reactive control	1 tube
RPR nonreactive control	1 tube

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-832-1
LABORATORY SCIENCES DIVISION 067
CLINICAL MICROSCOPY BRANCH

Mono-Chek Test

A. Procedure

1. Allow all reagents and specimens to reach room temperature.
2. Using dispenstirs, deliver one free-falling drop of serum specimen into the center of the circle on the test slide.
3. Using dropper from the bottle, deliver one free-falling drop of the Mono-Chek positive and negative controls into separate circles on the test slide.
4. Using the dropper, deliver one free-falling drop of thoroughly mixed Mono-Chek test red cell reagent to each specimen on the test slide.
5. Mix the serum-antigen suspension with a clean applicator stick and spread over entire circle.
6. Rock and rotate slide manually for 2 minutes.
7. At the end of the 2 minutes, observe for agglutination.

B. Interpretation

If agglutination is observed then infectious monucleosis antibodies may be present. If mononucleosis heterophile antibodies are not present then no agglutination occurs, and the result is reported as negative.

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*This mimeo supersedes M 08-280-824-5, 116.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-832-2
LABORATORY SCIENCES DIVISION 067
CLINICAL MICROSCOPY BRANCH

Rapid Slide Screening Test for Rheumatoid Arthritis

PRINCIPLE:

There is present in the blood plasma of arthritic patients a specific protein component known as the "Rheumatoid Factor." The serologic procedure for detecting the rheumatoid factor depends upon the interaction between this factor and gamma globulin from humans who do not have rheumatoid arthritis. When certain relatively large carrier particles are coated with normal gamma globulin and mixed with serum from arthritics, agglutination of the carrier particles occurs.

TECHNIQUE:

1. In a test tube, prepare an approximate 1:20 dilution of the serum under test by adding one drop of serum to one ml of glycine-saline buffer diluent.

NOTE: pH of this buffer must be carefully maintained at 8.2.

2. Using dispenstirs, place one drop of the diluted serum in one section of a divided glass slide.

3. Add one drop of latex-globulin reagent to the diluted serum and mix with the dispenstir. Spread the mixture over a circular area approximately 20 mm. in diameter.

4. Prepare positive and negative controls by placing one drop of the positive and negative control sera into the other two divisions on the glass slide.

5. Add one drop of the latex-globulin reagent to each of the controls and mix. Use a separate dispenstir for each mixture.

6. Tilt the slide from side to side for one minute and observe for macroscopic clumping.

7. A serum which contains the rheumatoid factor will produce visible flocculation of the latex-globulin particles. A smooth, even suspension is observed in a few seconds in negative tests and it remains smooth throughout. In positive tests, visible flocculation usually occurs in a few seconds.

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*This mimeo supersedes M 08-280-824-2, 103.

8. The results of the test should be reported as negative, weakly reactive, or reactive. In interpreting reactions the patient's serum should be compared with controls. The criteria for interpretation are as follows:

NEGATIVE---Smooth suspension with no visible flocculation. This is demonstrated by the negative control.

WEAKLY REACTIVE---Visible flocculation with small aggregates or partial clumping.

REACTIVE---Visible flocculation with large aggregates and complete clumping. The background is clear. This is demonstrated by the positive control.

INTERPRETATION:

A positive slide test indicates the presence of the rheumatoid factor but does not reveal the titer. If slide test results are positive, a tube test should be performed.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-832-3
LABORATORY SCIENCES DIVISION 067
CLINICAL MICROSCOPY BRANCH

Pregnosticon^R Rapid Slide Test

A. Procedure

1. After assembling the capillary pipet and bulb, draw up sufficient urine to the second mark on the pipet and discharge into circle on the test slide.
2. Using the dropper with the black bulb, squeeze one free-falling drop of anti-HCG onto the circle with the urine and mix with stirrer stick.
3. Mix for 30 seconds.
4. Shake up the latex particles coated with HCG and squeeze one free-falling drop onto the urine-anti-HCG suspension.
5. Mix to obtain a homogenous suspension and spread over entire circle. Rock slide for a minimum of 2 minutes.
6. After 2 minutes read suspension for agglutination.

B. Interpretation

A positive result means the presence of Human Chorionic Gonadotropin. A negative is the absence of Human Chorionic Gonadotropin.

Agglutination - Negative

No Agglutination - Positive

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*This mimeo supersedes M 08-280-830-2, 116.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-832-4
LABORATORY SCIENCES DIVISION 067
CLINICAL MICROSCOPY BRANCH

Rapid Plasma Reagin

A. Procedure

1. Using dispenstir draw up serum and dispense one free-falling drop to a circle on the 18 mm circle card.

2. Using the flat end of the dispenstir spread the specimen over the entire circle of the card.

3. After briefly mixing the antigen, dispense a drop to corner of card to rid needle of air bubbles and dispense one free-falling drop into each specimen on the card. Do not mix serum-antigen suspension since rotator performs this task.

4. Place card on rotator. Moisten sponge in cover and place on rotator. Twin rotator on by turning past 8 and back to 8.

5. At end of the 8 minute rotation, pick up the card and read for agglutination.

B. Interpretation

Agglutination is reported as a reactive and no agglutination is reported as a non-reactive. Slight roughness is considered non-reactive.

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*This mimeo supersedes M 08-280-827-3, 116.

SP4 Howard C. Reese

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-832-5
LABORATORY SCIENCES DIVISION 067
CLINICAL MICROSCOPY BRANCH

Qualitative Cardiolipin Microflocculation Test (CLMF)

PRINCIPLE: The cardiolipin antigenic particles in properly prepared emulsion appear as short, thin rods. When reaction takes place between this antigen and the antibody-like material (reagin), that results from an infection by *Treponema pallidum*, the particles clump together and flocculate.

PROCEDURE:

1. Inactivate clear serum in a water bath at 56° C for 30 minutes.

NOTE: Serum which has been inactivated more than four hours before testing should be reheated in the water bath at 56° C for 10 minutes prior to testing.

2. While the serum is inactivating, prepare the antigen emulsion as follows:

a. Pipet 0.4 ml of the buffered antigen diluent into a flat-bottomed bottle of about 30 ml capacity.

b. While vigorously rotating the bottle on a flat surface, add 0.5 ml of the cardiolipin antigen dropwise directly into the diluent. The antigen should be delivered within 5 to 10 seconds, with the last drop being blown into the diluent without touching the pipette to the solution.

NOTE: Rotation of the bottle is necessary to prevent auto-flocculation of the solution.

c. Continue rotating the bottle for an additional 10 seconds.

d. Pipet 4.1 ml of diluent into the bottle.

e. Stopper bottle and shake vigorously for approximately 10 seconds.

f. This amount of antigen emulsion (5 ml) is sufficient for the testing of approximately 250 sera.

NOTE: The antigen emulsion has a shelf-life for 24 hours. If the antigen emulsion is to be used for testing throughout the day, it should be stored in the refrigerator when not in use.

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*This mimeo supersedes M 08-280-827-2, 103.

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3. Into one well of a clean Boerner slide, pipette 0.05 ml of inactivated serum, distributing the serum evenly in the bottom of the well.

NOTE: Use one well per sample, putting serum in all wells before adding antigen.

4. Apply one drop of properly prepared antigen emulsion using a 2 cc syringe with a 18 gauge needle (with bevel removed). When this combination of syringe and needle are held in a vertical position during application of emulsion the proper size drop (1/60 ml) is dispensed.

5. Rotate the Boerner slide for 4 minutes using either the shaker table or the manual method. If the shaker table is used, it must rotate at 180 rpm; if done by hand, rotate the slide at 120 rpm, circumscribing a 2-inch circle.

6. Read the CLMF test with the low-power objective under dim light. Focus DOWN, reading the first layer that comes into focus.

7. Aggregation of particles into large or small clumps is interpreted as degrees of reactivity and reported as follows:

APPEARANCE OF TESTS	DEGREE OF REACTIVITY	REPORT AS
No clumping or very slight roughness	-	Non-reactive (NR)
Small clumps	± or 1+	Weakly Reactive (WR)
Medium and large clumps	2+, 3+ 4+	Reactive (R)

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-833
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Graded Serological Procedures (4 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Laboratory

REFERENCES: All previous references

STUDY ASSIGNMENT: Review all previous lecture material and mimeos

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor for 12 students

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; PA system with lapel mike.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: This graded exercise follows your practical work. You are now going to perform the procedures for rheumatoid arthritis, infectious mononucleosis, pregnancy and syphilis for grade.

B. Objective

1. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for rheumatoid arthritis to within at least 80% accuracy.

2. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for mononucleosis to within at least 80% accuracy.

3. Given serum specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for syphilis to within at least 95% accuracy.

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*This lesson plan supersedes LP 08-280-833, 067

4. Given urine specimens, a commercial test kit with manufacturer's directions, and procedural handouts, perform serological tests for pregnancy to within 80% accuracy.

C. Class Procedure and Lesson Tie-in

Relates to all previous material

II. APPLICATION (225 min)

A. Instructors' Activities

1. Before class prepare keyed specimens and controls according to Annex A.

2. Issue equipment and reagents according to Annex B.

3. Supervise students.

4. Allow sufficient time at end of graded exercise for storage of equipment, reagents, and specimens, and cleaning of work areas.

B. Students' Activities

1. Receive equipment, reagents, and specimens from instructor.

2. Perform procedures using M 08-280-832-3, M 08-280-832-4, M 08-280-832-5, and M 08-280-832-6.

3. Record results and hand in to instructor.

4. After completion, store reagents, equipment, specimens, and clean up work area.

III. SUMMARY (10 min)

A. Questions from Students

B. Review

1. Present observed problems

2. Present solutions to problems

C. Closing Statement: These graded exercised have shown you how well you perform the basic techniques for rheumatoid arthritis, mononucleosis, pregnancy, and syphilis.

INSTRUCTIONAL HOURS

Number of instructors required - - - - 1 per 12 students

Primary hours	3
Secondary hours	0
Total	3

ANNEX A
INSTRUCTIONAL AIDS

Preparation of Specimens

A. Rheumatoid Arthritis

1. Prepare sufficient 10 X 75 mm test tubes, numbered consecutively with "R" prefix (i.e., R1, R2, R3) to provide 10 samples per student plus 20 extra. Store labeled tubes in 90 hole test tube racks.

2. Remove, at random, a maximum of five tubes per each 15 tubes in the 90 hole rack; set these aside, recording the tube numbers. These tubes will be used to contain positive samples.

3. Perform a serial dilution on a bottle of RA positive control serum to determine the highest dilution which will yield an acceptable positive result. Make dilutions with glycine saline.

4. Count the number of positive tubes set aside and multiply this total by 2 to determine the total volume (in ml) of positive samples required for student samples. Add 10 ml to this figure to allow for spillage and/or replacement.

5. Pool sufficient bottles of RA positive control serum to yield the total volume (as determined in step 4 above) of the proper dilution (as determined in step 3 above).

6. Dilute the pooled control serum and test to insure that an acceptable positive reaction is obtained. Dilute with glycine saline and add thimerosal as preservative.

7. Prepare sufficient negative specimen to provide 2.0 ml for each negative sample tube. Use normal saline, adding bovine albumin to provide proper surface tension.

8. Use food coloring to adjust colors of all samples until they are visually similar.

9. Dispense proper specimens into proper tubes (2.0 ml/tube) and refrigerate.

10. Set up control serum for students, using the positive and negative specimens.

B. Infectious Mononucleosis

1. Prepare sufficient 10 X 75 mm test tubes, numbered consecutively with "M" prefix (i.e., M1, M2, M3, etc.) to provide 10 sample tubes per student plus 20 extra. Store labeled tubes in 90 hole test tube rack.

2. Remove, at random, a maximum of 5 tubes per each 15 tubes in the 90 hole rack; set these aside, recording the tube numbers. These will be used to contain positive samples.

3. Pool positive patient serum. (Positive serum for infectious mononucleosis should be obtained through liaison with local medical facilities).

4. Set up a twofold serial dilution on the serum and perform a slide test for mono on each tube.

5. Select the highest dilution which gives a distinct, readable positive reaction.

6. Dilute the pooled serum to provide 2.0 ml for each tube set aside in step 2 above, using the dilution selected in step 5 above.

7. Prepare sufficient negative specimen to provide 2.0 ml for each negative sample tube (those tubes remaining in the 90 hole racks). Use normal saline, adding bovine albumin to provide proper surface tension.

8. Use food coloring to adjust the colors of all samples until they are visually similar.

9. Retest the positive samples to insure that an acceptable reaction is obtained.

10. Dispense designated specimens into proper tubes (2.0 ml per tube) and refrigerate.

11. Set up control serum for students, using the positive and negative specimens.

C. Pregnancy

1. Using adhesive tape and ball point pen, consecutively number sufficient 10 X 75 mm test tubes to provide 10 tubes per student plus about 30 extra tubes.

2. Using 90 hole test tube racks, arrange the labeled test tubes in numerical order in groups of 90 tubes.

3. At random, remove a maximum of 5 tubes per each 15 tubes in 90 hole rack; set these aside to use as positive specimens.

4. Record the numbers of the positive specimens to use by the instructor as a key. Example:

Pregnancy Test Key

1-100 1, 2, 7, 14, 18, 21, 26, 33, 39, 47, 53, 58, 62, 69, 74, 75, 77, 83, 85, 87, 87, 94, 98, 100

5. Dilute HCG - positive urine samples with normal saline to prepare working urine specimen. The quantity prepared should be sufficient to provide 2 ml for each positive specimen tube. Check working urine specimen for reactivity, using slide test pregnancy. Use normal saline for negative specimens. Match colors of all specimens with food coloring to prevent color identification of specimens by students. Store specimens in a refrigerator.

6. Dispense 2 ml of the proper specimen into the proper tube set up in step 3 above.

7. Set up control urine for students, using the positive and negative specimens.

D. Syphilis

1. Prepare sufficient 10 X 75 mm test tubes, numbered consecutively with different colored tape to provide 10 samples per student with 20 extra.

2. At random, remove a maximum of 25 tubes from each group of 100. These tubes will be used as reactive specimens. All remaining tubes will be used for nonreactive specimens.

3. Record the reactive numbers to be used by instructor as a key.
1-100 R 1, 2, 7, 14, 18, 21, 26, 33, 39, 53, 56, 58, 62, 69, 70, 75, 79, 80, 83, 94, 97, 98, 99, 100

4. Titer the pooled control serum and dilute with normal saline to produce 250 ml of reactive specimen. Using 22% bovine albumin, insure that there is a protein concentration of about 2 gm % in the specimen. 20 ml of 22% bovine albumin in 250 ml of normal saline is sufficient. The protein is required to allow the specimen to spread out to the border of the 18 mm circle. Add albumin to normal saline to produce nonreactive specimens.

5. Check the specimens to insure their reactivity.

6. Dispense 2 ml of the appropriate specimen into each of the tubes set up in steps 2 and 3 above.

7. Refrigerate all dilutions and specimen tubes until needed.

8. Set up control serum for students, using the reactive and nonreactive specimens.

ANNEX B
INSTRUCTIONAL AIDS

Reagents and Equipment

A. Rheumatoid Arthritis	To each Student
RA Latex Globulin Reagent	1 Bottle
Glass Test Slide	2
Dispenstirs (to deliver 0.05 ml)	1 Box
Stirrers	1 Box
RA Positive control	1 tube
RA Negative control	1 tube
Kimwipes	1 Box
Glycine saline buffer	Not needed
B. Infectious Mononucleosis	
Horse RBC Antigen suspension	1 Bottle
Mono positive control	1 tube
Mono negative control	1 tube
Glass test slide	2
Stirrers	1 Box
Dispenstirs (to deliver 0.05 ml)	1 Box
Kimwipes (already issued)	1 Box
C. Pregnancy	
Anti-HCG	1 Bottle
Latex particles coated with HCG	1 Bottle
Glass test slides	2
Stirrers	1 Box
Capillary tubes (to deliver 0.03 ml)	1 Box
Bulb	1
Kimwipes (already issued)	1 Box
Pregnancy positive control	1 tube
Prengnancy negative control	1 tube
D. Syphilis (Rapid Plasma Reagin)	
RPR Rotator	1
RPR 18 mm circle	1 Box
RPR antigen	1 Bottle
20 gauge needle without bevel	1
Dispenstirs (to deliver 0.05 ml)	1 Box
Kimwipes (already issued)	1 Box
RPR reactive control	1 tube
RPR nonreactive control	1 tube

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Grading System for Rheumatoid Arthritis, Mononucleosis

1. Check students reports using the key made in Annex A. The following results are incorrect.

   a. Positive test reported as negative.

   b. Negative test reported as positive.

2. The student is required to perform tests on ten specimens. In order to pass this crucial objective, the student may make no more than two errors; this equates to a graded score of 70% and an accuracy of 80%. One additional percentage grade point is awarded if nine tests are performed correctly; two additional are awarded if all ten tests are performed correctly. Maximum achievement in this crucial objective is reflected in a graded score of 20 percentage grade points. If the student makes more than two errors, student will have to attend a reteach and retest of that part of the crucial objective.

   Example of Grading System:

Number of Errors	Graded Score
0	10
1	9
2	8
3	Fail

ANNEX C
INSTRUCTIONAL AIDS

Grading System for Syphilis

1. Check students reports using the key made in Annex A. The following results are incorrect.

   a. Reactive specimens reported as non-reactive.

   b. Non-reactive specimens reported as reactive.

2. The student is required to perform 100 tests. In order to pass this crucial objective, the student may make no more than five errors. If the student makes more than five errors, he or she will have to appear in the classroom for a reteach and retest on Syphilis testing.

   Example of Grading System:

Number of Errors	Graded Score
0	30
1	28
2	26
3	24
4	22
5	20
6	Fail

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ANNEX D
INSTRUCTIONAL AIDS

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Grading System for Pregnancy Tests

1. Same as Annex C

2. The student is required to perform tests on ten specimens. In order to pass this crucial objective, the student may make no more than two errors; this equates to a graded score of 70% and accuracy of 80%. Two additional percentage grade points are awarded if nine tests are performed correctly; four additional are awarded if all ten tests are performed correctly. Maximum achievement in this crucial objective is reflected in a graded score of 20 percentage grade points. If the student makes more than two errors, student will have to attend a reteach and retest of this crucial objective.

Example of Grading System:

Number of Errors	Graded Score
0	20
1	18
2	16
3	Fail

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MODIFICATIONS

LP 08-280-835 Examination of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material was not available at time of publication and its exclusion does not alter the value of remaining material.

Chapter 1

INTRODUCTION TO SEROLOGY

SECTION A—INTRODUCTION

1-1. Serology Defined. It is logical that the sooner an accurate diagnosis of a disease is made, the sooner appropriate therapy can be started. Consequently, much research, with many advances, has been directed toward more rapid and accurate diagnoses. One of the areas of clinical laboratory science that has been expanding most rapidly along these lines is clinical serology.

a. Basically, serology can be defined as the study of serum. More specifically, serology is diagnosis through the use or detection of serum globulins, known as antibodies. These antibodies may even have been produced in another animal and then used to diagnose disease in man. In effect, any in vitro diagnostic test using antibodies can be called a serologic test or serotest. However, antibodies are only half of the reactants in serotests. Antibodies must have something to react with—namely, antigens.

b. This manual is concerned with mixing these antigens with antibodies and reporting the resulting antigen-antibody reaction, if any, to the physician. General characteristics of antigens, antibodies, and their reactions are covered in the next chapter with the remainder of the manual devoted to laboratory techniques and serotests for specific diseases or conditions.

c. Due to the special techniques and time-consuming steps used in preparing them, many of the reagents used in serologic tests are available in kit form. Many kits are available through regular supply channels while others are available commercially. Attachment 3 is a partial list of sources of serologic kits and reagents. Some of these kits contain everything needed to accomplish the test. Consequently, relatively few items of glassware and laboratory equipment are used in serologic testing. In fact, more and more disposable items are being used. Glassware and equipment used in serotests are covered in the next two sections of this chapter.

SECTION B—GLASSWARE

1-2. Test Tubes. Several serotests are performed in test tubes. In addition, the patient's serum is the most frequently assayed specimen, thus requiring more test tubes. Those test tubes most commonly used are 12X75mm (Kahn), 13X100mm (Wassermann), and centrifuge tubes. Both reusable and disposable types are available through the supply system.

1-3. Slides. Many, in fact most, recently developed serotests are performed on glass slides or plastic-coated paper cards instead of in test tubes. These slide tests use very small quantities of reagents and, in addition, many use the disposable paper cards, thus making these tests very efficient for the busy laboratory. Other tests use just plain glass slides; black glass slides or tiles; slides with molded concavities; or slides with ceramic or paraffin rings. Each test kit usually comes complete with the recommended slide. Others must be purchased separately or prepared in the laboratory. Be sure to use the specific type of slide that is indicated for each given procedure.

1-4. Pipets. Serotests require the mixture of accurately measured volumes of reagents. Although many tests utilize medicine droppers to dispense reagents, even these droppers are manufactured to deliver a specific size of drop. Several serotests require larger volumes of reagents than can be delivered conveniently by droppers. For these tests, pipets are more efficient. Several reusable types are available.

a. Types of Pipets Used in Serology:

(1) Serological Pipets. Appropriately, the pipets most commonly used in serotests are serological pipets. Examples of common sizes are shown in figure 1-1. As indicated by the etched mouthpieces, these pipets are allowed to drain freely and, then, the last drop is blown out. Serological pipets are calibrated to deliver (T.D.) a given volume at a specific temperature, usually about 20°C. Therefore, for most accurate use, they should not be rinsed out, but this practice is, at times, ignored in serotesting. Serological pipets must not be confused with Mohr pipets which are also T.D. pipets. Mohr pipets are

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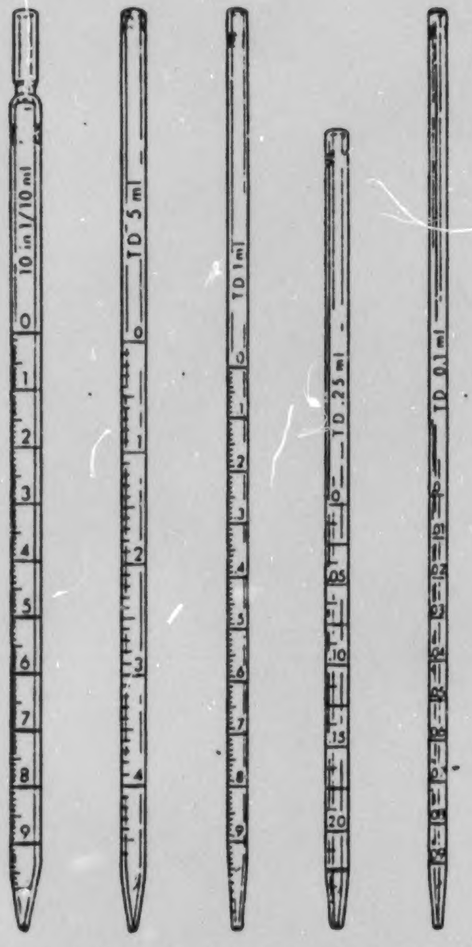
not calibrated to their tips and must be used only with the point-to-point pipetting technique. It is best to keep Mohr pipets out of the serology area.

(2) Capillary Pipets. These pipets are also called Pasteur or transfer pipets. They are most useful in transferring reagents, such as serum, from a clot to another tube. They work most efficiently with a rubber bulb but may also be filled by mouth. Two sizes of Pasteur pipets are available. In addition, they may be easily made from glass tubing by heating and drawing out the tubing.

b. Using Serological Pipets:

(1) Select the smallest pipet that will deliver the volume required. Use only clean, dry pipets without chipped tips. Those with chipped tips should be discarded.

(2) Draw fluid slightly above the desired mark, using the tip of the forefinger (index finger) to control the flow.



10.0 ml 5.0 ml 1.0 ml 0.25 ml 0.1 ml

Figure 1-1. Serologic Pipets.

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(3) Hold the pipet vertically and wipe the tip (wet area) with gauze or lint-free absorbent paper.

(4) Holding the pipet vertically so that the fluid level is at eye level and reading from the bottom of the meniscus, let the fluid down exactly to the desired mark. Do not put the tip of the pipet back into the fluid during this operation. Touch the tip of the pipet to the inner wall of container to dislodge any droplet that might be hanging from the tip.

(5) Recheck the fluid level. Repeat the previous steps if the fluid is not at the desired mark.

(6) Transfer the pipet to the intended container and let the fluid drain freely before blowing out the last drop. Do not immerse the tip while draining. If increased accuracy is desired, a point-to-point pipetting technique may be used but this is seldom required in serologic testing, unless Mohr pipets are used. The specialized use of serological pipets in making serial dilutions of serum is covered in chapter 4.

1-5. Cleaning Glassware. Most serotests are performed under closely controlled conditions of pH and ionic concentrations. Without prerinsing, even disposable glassware fresh from the manufacturer may not be clean enough for serotests. Although these tests rarely require sterile glassware, all reusable glassware that has been in contact with patient's blood or infectious organisms must be sterilized before cleaning to reduce the danger of transmitting infectious hepatitis and other infections. Methods for prerinsing and cleaning glassware are given below.

a. Prerinsing Glassware. For reusable glassware, the most useful way to assure cleanliness for future tests is to prerinse the glassware immediately after use. Of course, if live organisms are used, sterilize the items first. Assuming that no viable pathogenic organisms were used in the test, the simplest way to prerinse is with running tapwater followed by immersing the glassware completely in tapwater with or without a detergent. In other words, don't let the reagents dry in or on the glassware. Glassware containing relatively small amounts of reagents could be placed directly in the water without rinsing. In addition, pipets are especially inconvenient to rinse immediately after use, so these should be immersed completely in a vertical position in water containing detergent. This is best accomplished in a cylinder with a cotton-lined bottom to prevent chipping.

b. Cleaning Test Tubes and Slides:

(1) If desired, these items may be soaked overnight in hot tapwater containing a neutral detergent such as any of the well-known liquid household dishwashing detergents. Highly alkaline detergents should not be used for serologic glassware because of the difficulty in removing residual alkali that may affect test results. In addition, prolonged exposure to alkali will etch the glassware.

(2) Actual cleaning of the glassware can usually be accomplished with a nylon or similar brush in hot tapwater containing neutral detergent. Only excessively dirty items will normally have to be subjected to stronger cleaning solutions such as the chromic acid cleaning solution covered in paragraph 1–5f.

(3) Rinse the glassware several times in hot running tapwater and, then, several times in fresh distilled water.

(4) Dry in an inverted position in a drying oven (100°C) and store in a dust-free area. Be sure to discard chipped or cracked tubes. Using cracked centrifuge tubes could be especially wasteful of both time and reagents—not to mention the possible inconvenience to the patient.

c. Cleaning Larger Pipets. Pipets in sizes of one ml or larger can be cleaned efficiently in automatic pipet washers such as described in paragraph 1-6. Cleaning smaller pipets will be covered in paragraph 1–5d. Excessively dirty pipets may be cleaned in chromic acid cleaning solution.

(1) Sort the prerinsed pipets, removing those smaller than one ml.

(2) Make sure the tips are not clogged with debris.

(3) Place the pipets in the holder (basket) with tips uppermost.

(4) Place basket in washer and wash at least 2 hours. Most washers can be set to cycle at least 10 times per hour. Be sure to observe several cycles to be certain that the correct water pressure has been applied and that the washer is cycling properly. Do not simply turn on the water unless the pressure has been preregulated.

(5) Remove the pipets and recheck for clogged tips. Clogged pipets will not wash by this method.

(6) Allow the pipets to drain briefly and rinse 5-6 times in fresh distilled water, making sure to drain the pipets completely after each rinse.

(7) Dry in a hot air oven (100°C).

d. Cleaning Smaller Pipets. Pipets smaller than one ml will not flush adequately by gravity as employed in automatic pipet washers. Consequently, small pipets must be washed by aspiration using a vacuum such as produced by a faucet vacuum pump. Several adapters for various numbers of pipets are available. After rinsing for several minutes with tapwater, the pipets are rinsed with distilled water, drained, and dried in a hot air oven (100°C) or they may be dried by flushing with acetone followed by air.

e. Precleaning Disposable Glassware. Disposable test tubes are very convenient for serotests. However, most disposable glassware is made from very cheap glass. This glass may still contain contaminating alkali unless it has been precleaned at the factory at added cost. Because serotests are easily affected by chemical contamination, this alkali must be neutralized. Neutralization is accomplished by immersing the glassware overnight in 2% (v/v) hydrochloric acid. Then, the glassware is rinsed several times in hot running tapwater followed by fresh distilled water and dried.

f. Chromic Acid Cleaning Solution. Unusually dirty glassware may be cleaned by immersion in chromic acid cleaning solution. However, due to the difficulty of insuring adequate rinsing, serologic glassware should not be cleaned this way unless absolutely necessary.

(1) Preparing Chromic Acid:

Sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) or potassium dichromate ($K_2Cr_2O_7$), technical grade	100 g
Tapwater (or distilled water)	1000 ml
Sulfuric acid (H_2SO_4), concentrated, technical grade	1000 ml

Dissolve the dichromate in the tapwater in a large sturdy container. Once the dichromate is dissolved, place the container in a sink with cold circulating water covering the level of the liquid. While wearing rubber gloves, an apron, and safety glasses, slowly add the sulfuric acid with constant stirring. (ALWAYS add acid to water slowly with stirring.) The solution will change from orange to dark brown as acid is added. This solution is usable until it turns green. Glassware containing large amounts of serum or other organic substances will rapidly reduce the useful period of the solution. Therefore, prerinsing and at least partial drying of the glassware is advisable before cleaning in chromic acid solution.

(2) Using Chromic Acid Cleaning Solution. Prerinsed and partially dried glassware is immersed CAREFULLY in the acid while wearing rubber gloves and safety glasses.

Special care must be taken to slowly immerse pipets as they may spurt acid if immersed too rapidly. Be sure test tubes and pipets are completely filled. Let stand overnight; then, using the previously mentioned safety precautions, rinse the glassware thoroughly in hot running tapwater followed by distilled water, and dry at 100°C. Larger pipets can be rinsed in a pipet water after acid cleaning.

SECTION C—LABORATORY EQUIPMENT

1-6. Pipet Washer. One of the more useful pieces of equipment used in serology is a pipet washer. Various automatic models are available—some with built-in dryers. Most can wash about 200 1-ml pipets at one time. Cleaning is by surging water as the washer repeatedly fills and empties. Pipets smaller than 1 ml should not be washed in these washers because the surging action is hampered by the small bore of the pipets. In addition, care must be exercised in setting the water pressure of these washers so that an effective surging action is achieved. A typical pipet holder and washer are shown in figure 1-2.

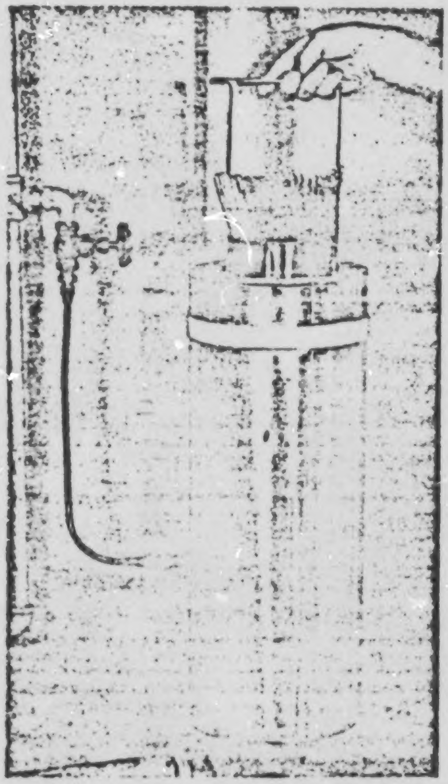


Figure 1-2. Pipet Washer.

1-7. Centrifuges. Centrifuges are used in serology mainly to separate patients' sera from blood clots. In addition, centrifugation is a step in the preparation of certain antigens, such as erythrocyte suspensions. Several types of centrifuges are applicable to serologic testing. Two examples are shown in figures 1-3 and 1-4.

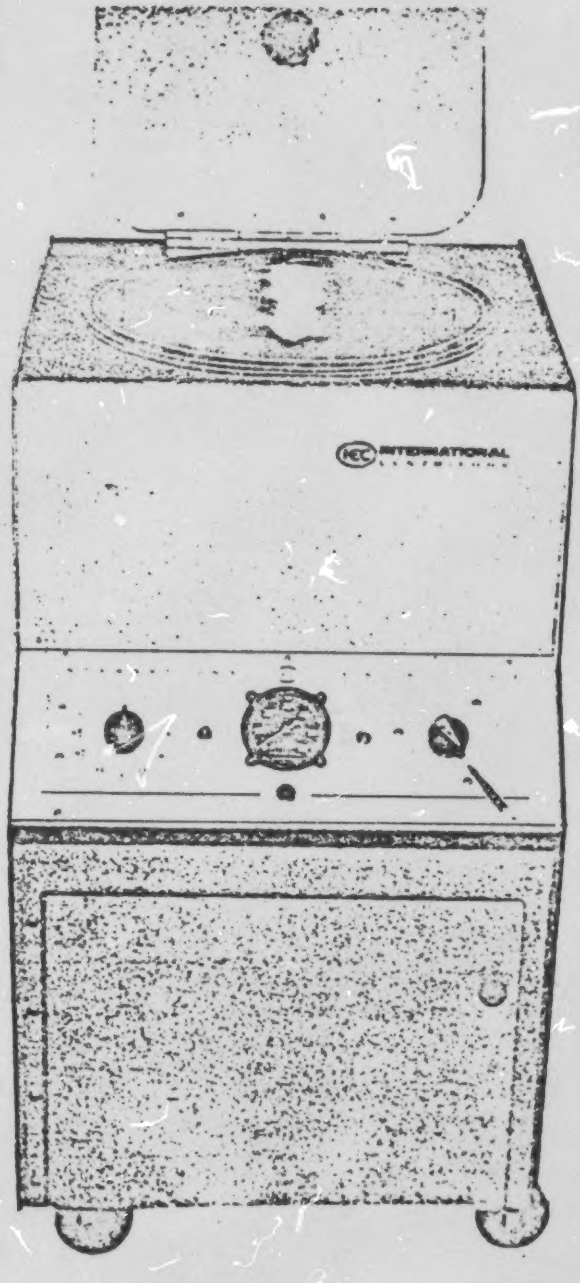


Figure 1-3. Centrifuge, Floor Model.

a. Use of Centrifuges. Usually each serologic procedure will specify the time and revolutions per minute (rpm) that centrifugation should be applied. These settings will be adequate for most general purpose centrifuges. However, certain procedures require very critical centrifugation. For these procedures, a specific relative centrifugal force (rcf) or gravity (g) is given. The rcf and g will be considered as synonymous for serologic purposes. Both rcf and g are based on rpm and the radius of the centrifuge. Although tachometers on centrifuges measure only rpm, most instruments will have conversion tables or nomograms to convert rpm to rcf (g). The required rpm for achieving a given rcf may also be calculated.

b. Calculating rpm From rcf (or g):

(1) Determine the radius (in centimeters) of the centrifuge head by measuring to the outermost TIP of the centrifuge tube. Centrifuges with swinging-type cups must have the cups extended horizontally before measuring the position of the tip of the centrifuge tube.

(2) Calculate the required rpm setting for the desired rcf using the following formula:

$$\text{Required rpm} = \frac{\text{Desired rcf (or g)} \times 105}{1.11 \times \text{radius (in cm)}}$$

c. Centrifugation Precautions. Valuable time and reagents can be lost if glassware shatters during centrifugation. In general, glass centrifuge tubes will not withstand relative centrifugal forces (rcf or g) of more than about 2000 X g. Therefore, know your centrifuge and when to switch to polyethylene or steel centrifuge tubes.

1-8. Rotating Machines. Slide rotators are invaluable for serotests that require prolonged rotation of the reactants on slides. Many serotests require closely controlled rotating speeds and times so machines with variable speeds and timers are especially useful. Be sure to calibrate rotators for each procedure. An example of a slide rotator is shown in figure 1-5.

1-9. Constant Temperature Devices. Several serologic tests must be performed or incubated at some constant temperature, usually body temperature. In these tests, a variation of 1°C from the prescribed temperature may not be acceptable. In other tests, a range of several degrees may still yield valid results. Four main types of constant temperature devices are useful in serology.

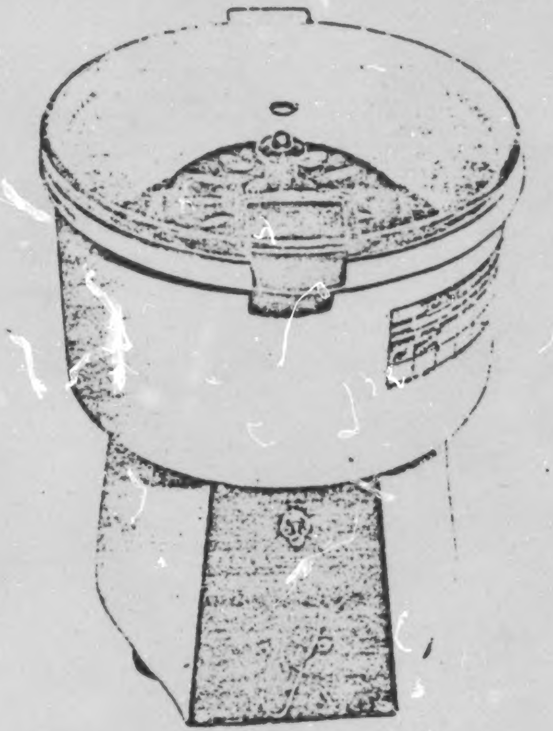


Figure 1-4. Centrifuge, Table Model.

a. Freezers. The best way to preserve serum, if it cannot be tested immediately, is to freeze it at minus 20°C or colder. This is because storage of serum at room temperature may allow microorganisms to denature the antibodies and, thus, render the serum useless for serotesting. Be sure to check storage instructions before freezing serologic reagents, because some reagents are destroyed by freezing.

b. Refrigerators. Refrigerators set at 1-6°C are used to store many serologic reagents. In addition, serum may be stored for short times at this temperature. Only two serotests, the cold hemagglutination and CRP tests, are performed in a refrigerator.

c. Water Baths. Variable-temperature water baths are available that can maintain temperatures within 0.5°C of any desired temperature from slightly above room temperature up to about 60-70°C. Although heat transfer to test tubes immersed in water is excellent, these tubes must be handled carefully as dripping water could ruin a test. A typical water bath is shown in figure 1-6.

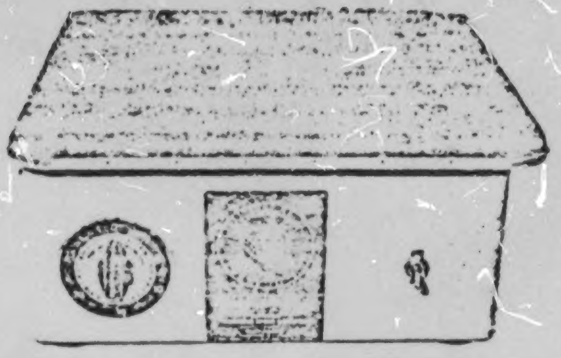


Figure 1-5. Slide Rotator.

d. **Dry-Heat Blocks.** Heating blocks can maintain temperatures as constant as a water bath and are useful for temperatures including boiling and higher. However, heat transfer is not as efficient as with a water bath and test tubes exactly the diameter of the heating block must be used. Fortunately, units with interchangeable tube holders are available. See figure 1-7 for an example of a dry-heat block.

1-10. **Microscopes.** Microscopes are required to read certain antigen-antibody reactions that do not yield macroscopically visible reactions. Two main types of microscopes are used in serology.

a. **Light Microscopes.** The use of ordinary light (compound) microscopes is declining rapidly in serology as more and more macroscopic tests are developed. However, certain tests for syphilis still rely on light microscopes. The specific use in these tests is covered in chapter 6. Information on the care and use of light microscopes may be found in AFM 160-51, Clinical Laboratory Procedures—Hematology.

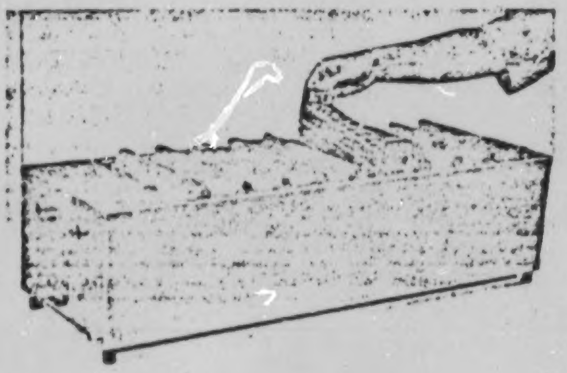


Figure 1-6. Water Bath.

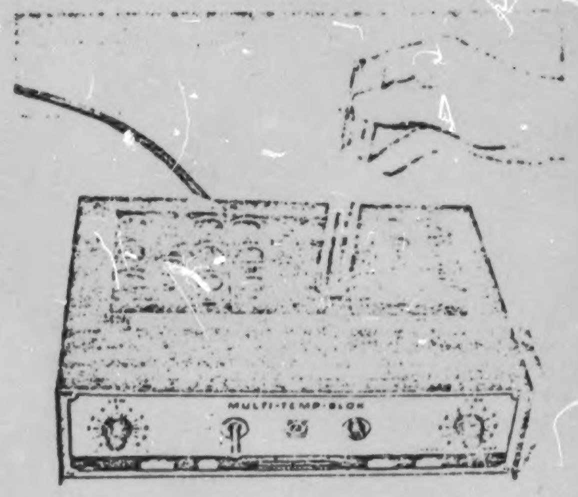


Figure 1-7. Dry-Heat Block.

b. **Fluorescent Microscopes.** Fluorescent microscopes are basically light microscopes that have a system of barrier filters built into their barrels. Most of these microscopes can still be used as light- and dark-field microscopes too. The important difference with a fluorescent microscope is the light source.

(1) The light is usually from a high-pressure mercury vapor bulb that emits light rich in wavelengths of 300-400nm—the upper UV area. A system of filters removes most other wavelengths, thus exposing the object under study to these UV wavelengths. If the object is stained with certain fluorescent dyes, the object will emit light of a color that is characteristic of the dye.

(2) After passing the object, extraneous UV light is filtered by the barrier filters in the barrel of the microscope. These filters serve two purposes. First, they protect the eyes of the observer. Second, allow the object to be seen in its characteristic colors against a dark background. Use of a dark-field condenser also enhances this contrast, but some procedures use bright-field condensers.

(3) The principle behind the use of fluorescent microscopes in immunofluorescence (fluorescent antibody) testing is covered in paragraph 2-12. Specific applications of the technique covered in this manual are in the fluorescent treponemal antibody-absorption (FTA-ABS) test in chapter 6 and the antinuclear antibody (ANA) test in chapter 12. An example of a fluorescent microscope with its power supply and light source is shown in figure 1-8.

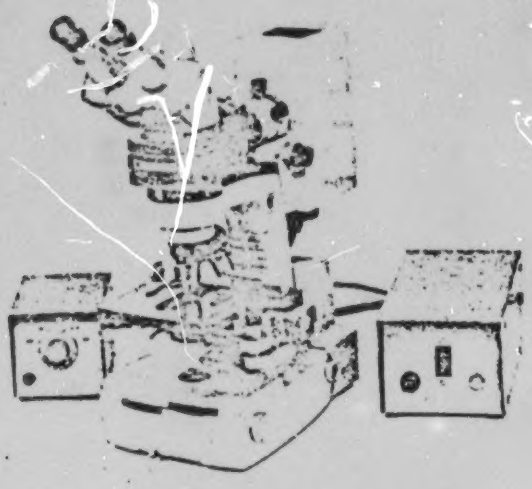


Figure 1-8. Fluorescent Microscope Apparatus.

1-11. Other Equipment. Several other items of equipment are used in serologic testing. Some are used only on rare occasions or only in larger or specialized laboratories. These will be discussed briefly.

a. Timers. Most serologic tests must be carefully timed. Several types of timers are available, some of which can be used to start or stop other equipment. In addition, many pieces of equipment, such as centrifuges, rotators, et cetera, may have built-in timers.

b. Sterilizers. Sterile glassware is rarely needed for serotests. Only tests that are incubated at body temperature for several hours would require sterile conditions. Of course, any equipment or glassware contaminated with viable organisms must be sterilized before being discarded or cleaned.

c. Microtitration Equipment. The development of microtitration equipment has proven to be a very useful substitute for test tubes and pipets in certain serotests. At times these tests use as little as one-tenth the amount of reagents as the original test method using test tubes, et cetera. In addition, microtitration is many times faster in setting up a test procedure than other methods. The basic equipment consists of calibrated microdroppers, plastic plates with wells in them, and the microdiluters. These items are available commercially. They are shown diagrammatically in figure 1-9. Many accessories. including automatic pipetters and diluters, are available to complement these items. Although microtitration equipment is required for only one procedure in this manual (the rubella titer in chapter 14), the technique has been applied to several other serotests. Examples are the antistreptolysin-O (ASO) test in chapter 9, tests for bacterial agglutination as in chapter 5, complement fixation tests, and others.

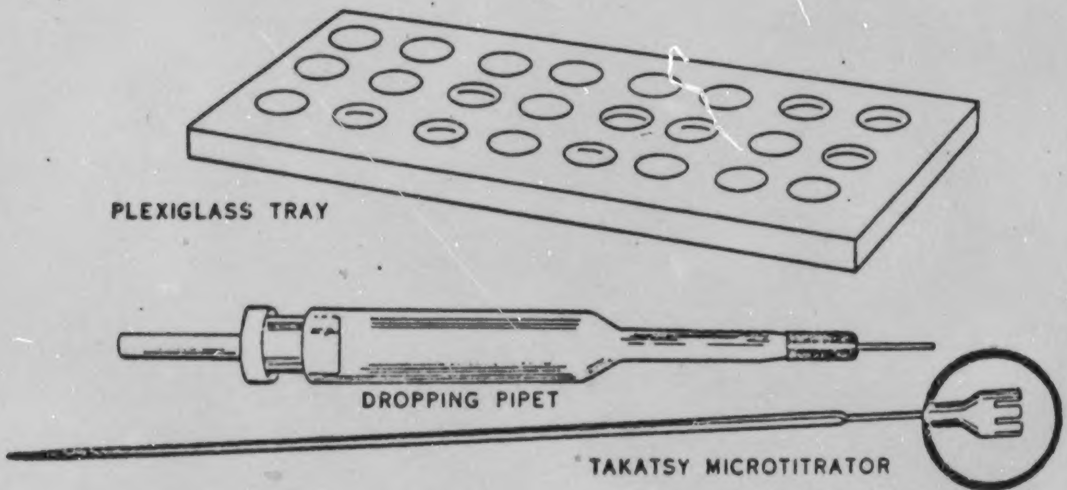


Figure 1-9. Microtitration Apparatus.

Chapter 2

IMMUNOLOGICAL BASIS FOR SEROLOGIC TESTS

SECTION A—IMMUNITY

2-1. Introduction. Resistance or immunity to disease can be classified under two broad headings—innate immunity and acquired immunity. If the disease-resisting ability is an inherited characteristic, the individual is said to possess innate immunity. Innate immunity is usually very general in action and not directed specifically at a given pathogenic organism. In contrast, acquired immunity is directed against a specific microbe or microbial product.

a. The specificity of acquired immunity involves inactivation of the pathogen or foreign substance by specific globulins, known as antibodies, that have been produced by the body in response to the pathogen or some foreign substance gaining entry into the body. These antibodies are found in body fluids—especially blood. Antibody production can also be stimulated by the injection of killed or attenuated organisms used as vaccines. Even parts of organisms or their toxins may stimulate antibody production. Immunity can also be acquired by producing antibodies in one individual or an animal and then injecting these antibodies into another individual, thus hopefully protecting the recipient.

b. Although all immunity involving antibodies may be classified as acquired immunity, not all antibodies are of a protective nature. Many antibodies arise from contact with foreign substances that are not connected with disease at all. In some diseases, the mere presence of certain antibodies, whether protective or not, is diagnostic. In other diseases, an increase in antibody level is significant. The detection and study of antibodies and their reactions provide the basis for the science of serology.

2-2. Innate Immunity. Innate immunity is the type where man is immune to canine distemper, while dogs do not contract syphilis. Each species has innate immunity to that particular disease. There are many degrees of innate immunity displayed. All species do not possess the absolute immunity described in the example. Even members of the same species vary in their ability to resist a given disease. Many defense mechanisms are active in innate immunity. Although there is much overlapping, these defense mechanisms can be divided into two categories—anatomical and chemical, excluding antibodies. A few of these will be described.

a. Anatomical Defense Mechanisms:

(1) Skin. The intact skin is an effective physical barrier to most disease-causing organisms. In addition, chemical mechanisms also operate here because the acid pH of the surface of the skin inhibits many microbes.

(2) Phagocytes. Throughout the body there is found a wide variety of cells which actively engulf (phagocytize) foreign particles, including microbes. White blood cells are well-known as phagocytes. Kupfer cells of the liver and microglial cells of the brain are examples of phagocytes which are localized in specific organs. As usual, chemical mechanisms cannot be excluded, for even the actual attraction of the phagocyte to the foreign particle is chemically or ionically mediated. Also, chemical degradation of the microbe takes place in the phagocyte if the phagocyte has the appropriate enzyme systems to destroy that particular microbe.

(3) Mucus. Mucus may act as a physical "trap" for disease agents. In combination with other mechanisms such as the cough reflex and cilia of the respiratory tract, mucus becomes an effective factor in preventing disease. Mucus can also contain phagocytes, enzymes, and antibodies which add effectively to disease resistance.

(4) Mouth. The simple act of swallowing moves potential pathogens to the stomach where few can survive. Coughing followed by swallowing aids in the removal and destruction of respiratory pathogens. Many enzymes and other microbial inhibitors are active in the salivary juices of the mouth too.

(5) Urinary Tract. The flushing effect of the urinary tract helps to physically remove potential pathogens.

(6) Eyes. The flushing action of tears, along with the presence of enzymes in the tears, provides one of the best examples of innate immunity. Acquired immunity is of minimal importance here. Even so, the eyes

are perhaps the most healthy organs of the body when one considers infectious disease.

(7) Lymphatic System. This system of ducts running throughout the body has nodes at intervals. These nodes can physically filter microbes from lymphatic fluid. Also, the nodes are the sites of accumulation of white blood cells, including fixed tissue macrophages. These cells are then in an ideal position to phagocytize the offending microorganisms.

b. Chemical Defense Mechanisms, Excluding Antibodies:

(1) Lysozyme. This enzyme is present in many body fluids such as mucus, tears, and saliva. Primarily, it attacks the cell walls of gram-positive bacteria.

(2) Interferon. This protein is produced by the body in response to a viral infection or after the injection of dead viruses. It differs from antibodies in that interferon may provide protection against challenge by another completely unrelated virus while antibodies normally act only against the specific injected virus.

(3) Properdin. This high-molecular-weight protein is effective against certain viruses and also against some gram-positive bacteria and even a few gram-negative ones. Properdin might be considered to be an antibody-like substance as its action requires the presence of complement and magnesium ions, both of which are active in certain antibody reactions. In fact, properdin has been called a "natural antibody," but it lacks the specificity of an antibody and attacks many different organisms.

(4) Inflammation. Invasion by certain microbes causes inflammation which brings several defense mechanisms into action. For one, the temperature at the site of the inflammation or even throughout the body may rise, thus becoming a less favorable environment for many pathogens. The increased permeability of blood vessels at the site allows antimicrobial factors and white blood cells to readily contact the infectious agent. Alteration of the chemical composition at the site also acts as a defense mechanism. For example, the pH goes down as lactic acid and carbon dioxide accumulate. All of these changes during inflammation combine to resist further reproduction and dissemination of the pathogen.

2-3. Acquired Immunity:

a. Active Immunity. Active immunity is an acquired immunity because it involves specific antibodies against a microorganism or foreign substance. It is called active immunity because the individual actively produced these antibodies himself at some stage in his life. The antibodies may arise as a result of a clinical or subclinical infection by the given microbe. They may also be produced in response to vaccinations with killed or attenuated organisms. In either case, the individual produces the antibodies. Antibodies produced following actual infection are said to confer naturally-acquired active immunity. Vaccination produces artificially-acquired active immunity. The resulting antibodies may contribute to immunity in several ways or not at all. Some may cause a microbe to be more readily phagocytized, destroy it directly, neutralize some toxin produced by the organism, or combine these actions. Man may also produce antibodies that have no apparent protective value at all but their detection may be useful diagnostically.

(1) Naturally-Acquired Active Immunity. The fact that a person has recovered from an infectious disease does not guarantee resistance to another attack by the same microbe. For example, influenza and gonorrhea result in very short-lived immunity and repeated attacks are common. On the other hand, a single infection of measles or chicken pox usually confers life-long immunity to reinfection. There are other examples of these extremes of immunity and also many that fall between the extremes.

(a) Especially significant to the serologist is the rise in antibody titer during the course of a disease. In many cases, serological tests are the only practical diagnostic tools available. Perhaps isolation of the causative organism would be impossible, too expensive, or take too long to benefit the patient.

(b) Another important aspect of serological testing is the determination of antibody levels at a given time. A good example of this is the testing of serum from pregnant females for antibody titers against rubella (German measles). In this case, a certain titer usually indicates that the pregnant woman is immune to the rubella virus. Consequently, the safety of the developing fetus will most likely be assured in the event the immune woman should become exposed to rubella.

(2) Artificially-Acquired Active Immunity. For many diseases, vaccination has proven to be an effective method of providing immunity. This immunity is also not absolute and the relative efficiency of each vaccine can be guessed by the frequency that

reimmunization is required. In endemic areas, cholera and plague shots are given every 6 months, yellow fever shots, every 10 years. Obviously, the yellow fever vaccine is much more effective than cholera and plague vaccines. Although antibodies produced as a result of vaccination may benefit the patient, these same antibodies usually confuse the serologist. His tests usually will not differentiate between artificially-acquired antibodies and those produced in the presence of active disease. Consequently, a negative history with a rising antibody titer becomes very important in serodiagnosis. Of course, the problem is minimized in diseases where vaccination is not practiced.

b. Passive Immunity. Passive immunity is another type of acquired immunity because antibodies are involved. It differs from active immunity by the fact that the antibodies are produced in another individual or animal and then injected into the recipient. The recipient passively receives the immunity conferred by these antibodies. Although passively-transferred, antibodies are important to the recipient; they are short-lived and rarely assayed by the clinical serologist. As with active immunity, passive immunity may be acquired both naturally and artificially.

(1) Naturally-Acquired Passive Immunity. This type of immunity is significant mainly in the survival of the newborn infant. The infant passively acquires antibodies from its mother. The antibodies may pass from the immune mother to the fetus across the placental barrier. In addition, the infant may acquire these antibodies from its mother's milk which is rich in antibodies for a short time after birth. Of course, immunity is transferred only if the mother is immune to a given disease. Passive immunity is especially important to the newborn because they are incapable of producing antibodies of their own for a few months after birth. The antibodies received via natural transfer from the mother are relatively short-lived with protection seldom exceeding about 6 months. Fortunately, by this time the infant's immunological system is fully functional.

(2) Artificially-Acquired Passive Immunity. Antibodies that have been produced in another individual or animal and then administered by injection to the recipient provide this type of immunity. This method has been used extensively in the past in the treatment of diphtheria and tetanus through the injection of antibodies produced in horses. Before the advent of antibiotics, passively-administered antibodies were used as the treatment for pneumococcal pneumonia. Currently, passive immunization is mainly used for prophylaxis following exposure to such diseases as rubella and infectious hepatitis. This is usually accomplished by injecting the recipient with antibodies (gamma globulin) which have been extracted from the blood of immune persons. These antibodies provide protection for a relatively short time and then are removed from the system.

SECTION B—ANTIGENS AND ANTIBODIES

2-4. Antigens:

a. Introduction. Any foreign substance that stimulates the body (or any animal's body) to produce antibodies is called an antigen. To qualify as an antigen, these foreign substances must also react in some specific way with the antibodies that have been produced. An antigen might be a microbe, part of a microbe, some microbial toxin, or even some foreign product completely unrelated to microorganisms and the diseases they cause. Although antigens comprise a wide variety of substances, they have several characteristics in common. All function together to qualify a substance as an antigen.

b. Characteristics of Antigens:

(1) Foreign to the Body. Normally, the body does not respond by producing antibodies against itself. A notable exception to this is the production of antibodies against the lens of the eye. This may be explained by the fact that the lens is not in intimate contact with the reticuloendothelial system which produces the antibodies so the lens is, therefore, recognized as foreign. In addition, there are other examples of so-called autoimmune diseases in which the body produces antibodies against itself but these are usually associated with some malfunction of antibody production or antigen recognition. In general, however, the reticuloendothelial system responds only to antigens that are foreign, but even members of the same species have many antigens that are foreign to other members of the species. In man, the red blood cell antigens with their significance in blood transfusions and erythroblastosis fetalis provide good examples of foreignness within a species. Another example is seen in kidney transplantations, where usually only kidneys from very close relatives are accepted by the recipient. In time, kidneys from unrelated donors are usually rejected due to reaction with antibodies produced against kidney antigens. However, the mere fact that a substance is foreign to an individ-

ual does not confer antigenicity. Other characteristics must be present too.

(2) Molecular Weight. Another characteristic of antigens is that they are generally complex chemicals of very high molecular weight. A molecular weight of 10,000 is considered to be the minimum, but antigens with a molecular weight down to about 3,000 have been known. Some antigens have molecular weights in the millions. In general, the strongly antigenic substances have higher molecular weights than weakly antigenic substances.

(3) Chemical Nature. Most antigens are proteins or proteins complexed with carbohydrates or lipids and, as such, are derived from living organisms. As a general rule, carbohydrates and lipids are not antigenic unless in a protein complex. Only humans and mice appear to be able to produce antibodies against pure carbohydrates, such as certain polysaccharides, but this is a rare occurrence in nature.

(a) Each antigen has two parts—a carrier and a determinant. The carrier is usually a protein. Aside from contributing most of the molecular weight and increasing the rigidity of the antigen, the carrier functions mainly as its name implies—a carrier. The determinant is the important part of an antigen. It makes an antigen a specific, foreign substance. Although the determinant cannot stimulate antibody production by itself, it can react with the antibodies once they are produced. A determinant functioning in this fashion without its normal carrier is called a hapten. Many consider determinants and haptens as synonymous.

(b) Determinants (or haptens) comprise only a small portion of the total antigen molecule. For example, even the stereoisomers, D-tartaric acid and L-tartaric acid (M.W. 150), when conjugated with a carrier protein, form specific antigens. Each stimulates the production of antibodies which react only with the given isomer. In addition to lending specificity to the antigen and stimulating the body to produce specific antibodies against them, determinants are the reaction sites for the antibodies in antigen-antibody reactions. Each antigen molecule usually has about a dozen of these reaction sites (determinants) but some molecules may have a hundred or more. By contrast, the most common type of antibody molecule has only two reaction or combining sites while other types may have up to ten sites. Therefore, most antibodies are said to be bivalent, while antigens are multivalent. The valence of antigens and antibodies is especially important to the serologist in mixing the proper proportions of each in serologic tests.

(4) Reactivity with Antibodies. The last characteristic that an antigen must possess is that it must be able to react in some demonstrable way with the antibodies which have been produced in response to that antigen. The utilization and observation of these antigen-antibody reactions provide the basis for serological diagnosis and are covered later in this chapter and throughout this manual.

c. Fate of Antigens. Antigens may enter the body in a variety of ways. They may be acquired during the normal course of living, during disease, or following vaccinations. They may also be injected accidentally such as during a blood transfusion. Once they enter the body, they are very rapidly localized in the fixed macrophages of the liver, spleen, and bone marrow. The macrophages apparently process the antigen in some way, but the lymphocytes actually produce the antibodies. Some antigens are physically present in the macrophages during the time that the lymphocytes are actively producing antibodies. Whether this is necessary for continued antibody production is under investigation. Many antigens appear to be present for several months after injection; so retention may be essential for antibody production to occur.

2-5. Antibodies:

a. Introduction. Just as antigens are defined in terms of their reactivity with antibodies, all antibodies are intimately associated with their antigens. These antibodies must be able to react in some demonstrable way with the antigen which stimulated their production. Antibodies of significance to the serologist are found in the serum (or plasma) fraction of blood, but there are some antibodies that remain fixed to certain tissue cells. These fixed antibodies are of minimal significance to the serologist although their importance to the health of the individual should not be discounted. Whether fixed to cells or free in the serum, antibodies have several characteristics in common.

b. Characteristics of Antibodies:

(1) Specificity. In general, each antibody will react only with the antigen that stimulated the body to produce that antibody. In other words, each antibody possesses a high degree of specificity. As with any biological system, there are exceptions to the rule. These exceptions will be discussed later un-

der antigenic variation and as they apply to each test procedure.

(2) Chemical Nature. Antibodies are found in the globulin fraction of serum protein. More specifically, most antibody activity is in the gamma globulin fraction of serum globulin. Because these globulins are active in immunity, they are frequently called immunoglobulins as a synonym for antibodies.

(a) The immunoglobulins (antibodies) of man can be further classified based on the fact that they are antigenic themselves when injected into certain animals. The animals produce antibodies which precipitate the human immunoglobulins. Five main classes of immunoglobulins have been identified in man by this method. They are IgG, IgM, IgD, IgA, and IgE. Most antibody activity in human serum is due to IgG, IgM, or IgA with certain diseases causing the production of more of one class than another. For example, antibodies against bacterial endotoxins are primarily IgM, while those against the mumps virus are IgG. In addition, the class that is produced in greatest quantity varies with the number of times the individual has had contact with a particular antigen. Usually on the first contact, such as a vaccination, IgM is the main antibody produced; but on a second or subsequent contact, IgG usually predominates.

(b) The five classes of immunoglobulins also vary somewhat in molecular weight and structure. All are very large molecules with most classes having a molecular weight of 150,000 to 200,000; however, the molecular weight of IgM is about 900,000. Each immunoglobulin molecule consists of a combination of two types of chains of amino acids. These chains have been designated as light chains and heavy chains. The most abundant immunoglobulin molecule, IgG, is made up of two light and two heavy chains. An IgM molecule has ten light and ten heavy chains. Each chain of the molecule has a region with a constant amino acid sequence and a variable region. It is the variable region of the antibody molecule that was produced in response to a specific antigen. The variable region is also the binding site in antigen-antibody reactions. Figure 2-1 shows a schematic structure of an antibody (IgG) molecule.

2-6. Antigenic Sharing (Cross-Reactivity):

a. Introduction. So far, antigens, antibodies, and their reactions have been described as very specific entities. However, there are three main factors which serve to destroy the totality of the preceding discussion. First of all, a given organism, such as a bacterium, may possess several entirely different antigens, each of which stimulates the produc-

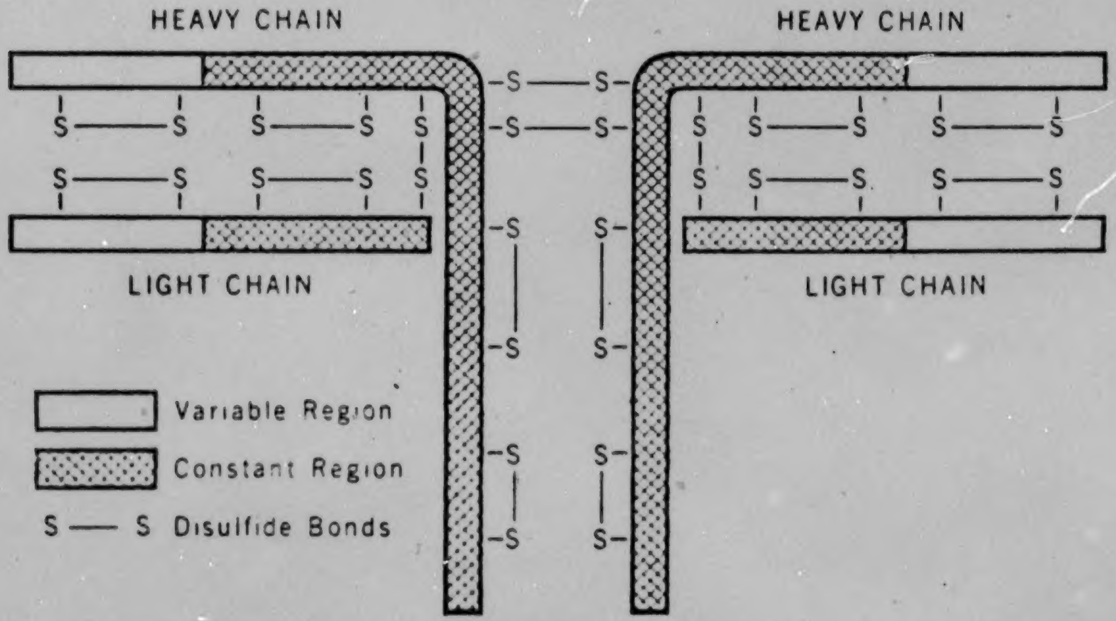
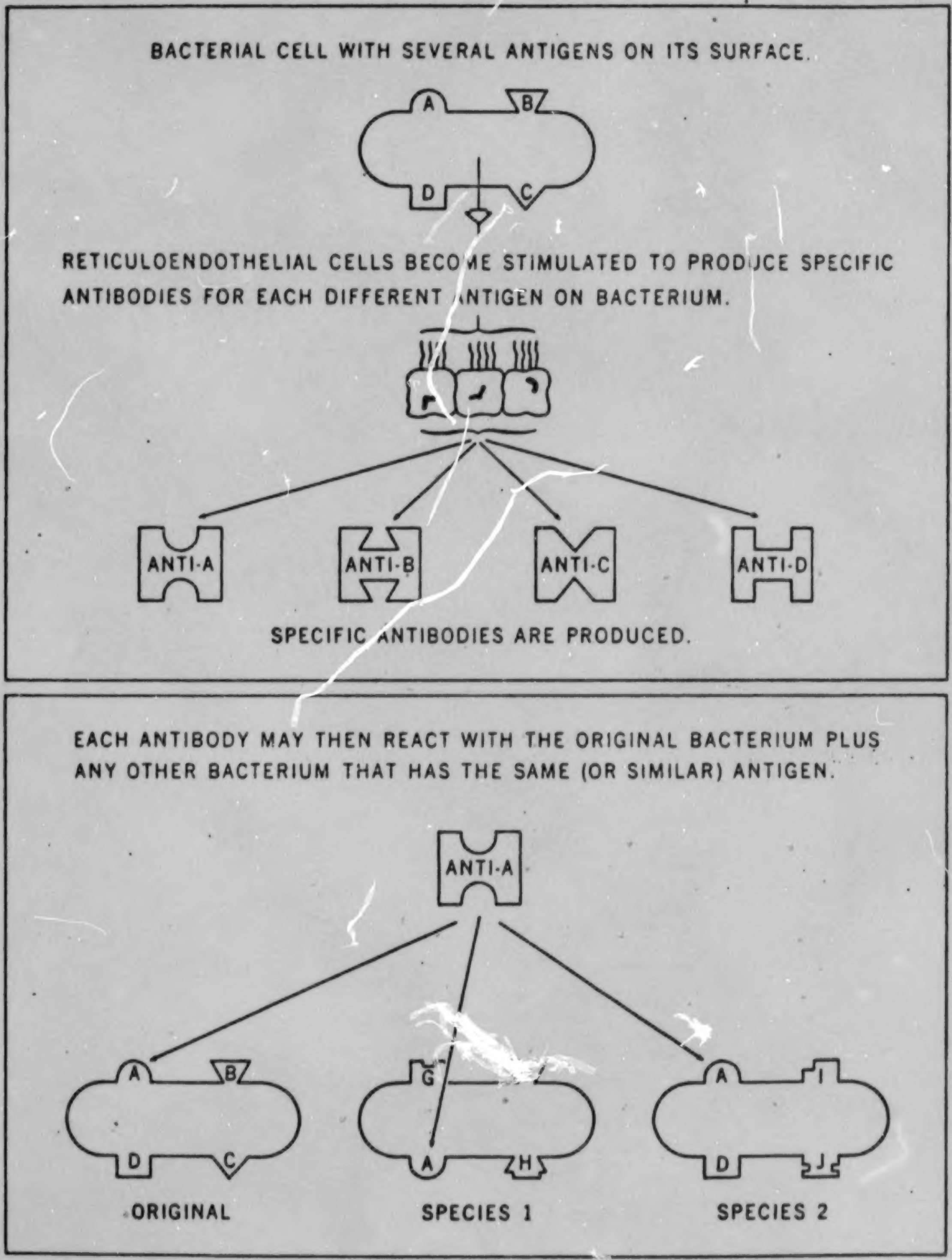


Figure 2-1. Schematic Structure of an Antibody (IgG).



BACTERIAL CELL WITH SEVERAL ANTIGENS ON ITS SURFACE.

RETICULOENDOTHELIAL CELLS BECOME STIMULATED TO PRODUCE SPECIFIC ANTIBODIES FOR EACH DIFFERENT ANTIGEN ON BACTERIUM.

SPECIFIC ANTIBODIES ARE PRODUCED.

EACH ANTIBODY MAY THEN REACT WITH THE ORIGINAL BACTERIUM PLUS ANY OTHER BACTERIUM THAT HAS THE SAME (OR SIMILAR) ANTIGEN.

A
B
D
C
ANTI-A
ANTI-B
ANTI-C
ANTI-D
ANTI-A
A
B
D
C
G
A
H
A
I
D
J
ORIGINAL
SPECIES 1
SPECIES 2

Figure 2-2. Cross-Reactivity.

tion of antibodies against that antigen. Secondly, evolution has resulted in seemingly unrelated organisms which may possess antigens in common with each other or even several organisms. Finally, the number of possible antigens (compounds) is limited by the number of elements occurring in nature so that duplications are inevitable. The end result is the production of antibodies which will react with identical or nearly identical antigens from two or more completely unrelated organisms. See figure 2-2. In serologic testing, this antigenic sharing results in what is referred to as cross-reactivity.

b. Significance of Cross-Reactivity. To the serologist, cross-reactivity is both a useful and, at times, a very confusing occurrence. The serologist in performing his tests must be constantly aware of the possibility that some completely unrelated antigen, such as a nonpathogen, may have stimulated the production of the antibodies under study. He must learn to rule out these so-called "false positive" reactions. At times this poses an insurmountable obstacle. On the other hand, the principle of cross-reactivity provides the basis for many useful serological tests. For example, patients with the supposedly viral disease, infectious mononucleosis, produce antibodies against sheep and horse red blood cells. This fact is then used in the laboratory diagnosis of infectious mononucleosis because sheep or horse red blood cells are more readily available and easier to use then the causative virus. In fact, no practical test is available for the detection of the infectious mononucleosis virus. Another good example is seen with rickettsial diseases, such as typhus and Rocky Mountain spotted fever. These rickettsiae have some antigens in common with certain bacteria of the genus *Proteus*. Since bacteria are more readily cultivated than rickettsia, the more economic route is selected by using bacterial antigens (*Proteus sp.*) in serologic testing for the unrelated rickettsial diseases. These are but two examples of the beneficial use of cross-reactivity by the serologist. Many more uses of cross-reactivity are seen in serologic testing including the most common of serologic tests—the ones for syphilis. Additional uses of cross-reactivity will be described as they apply to the procedures presented in this manual.

SECTION C—ANTIGEN-ANTIBODY REACTIONS

2-7. Introduction. Although in vivo antigen-antibody reactions are important to the health of a patient, the clinical serologist uses only in vitro reactions. After mixing antigens and antibodies in test tubes or on slides, the serologist must evaluate the reaction he observes, if any, and report his findings to the physician. Many times the mere presence or absence of a reaction is adequate for the physician, but at times an estimation of the antibody level is required. This is especially important in following the course of a disease where a change in antibody levels may be significant. Actually, no practical method is available for directly measuring antibody levels. However, analyzing the reacting capacity or titer of antibodies in a given serum provides a practical measure of antibody levels. This is usually accomplished by making serial dilutions of the patient's serum and noting the greatest dilution that still gives a positive reaction. The titer is then expressed as the reciprocal of the highest serum dilution to give the positive reaction. For example, if a serum dilution of 1/1280 was the highest to react, the titer would be reported as 1280 or 1:1280 against that antigen. This titer, while not an absolute measure of antibodies, provides a mathematical base for future tests on the same patient.

a. Sources of Antigens and Antibodies. As mentioned above, in many serologic tests the serologist will be trying to detect, and perhaps titer, antibodies in a patient's serum. He will accomplish this by reacting the patient's serum with some known antigen. However, in a few tests, he will be testing for the presence of a given antigen from man by using known antibodies (antisera) that have been produced in some other animal, such as a rabbit. Except for these antigens of human origin, antigens used in serologic tests come from a variety of sources. As mentioned in the discussion of antigenic (cross-reactivity), very useful serologic tests have been devised using antigens from sources which are apparently completely unrelated to the disease process under study. In addition, many antigens have been modified by the serologist in order to make the reactions more readily visible, increase the sensitivity of a test, or enhance the test in some practical way.

b. Stages of Antigen-Antibody Reactions. In vitro antigen-antibody reactions usually occur in two stages. The first is a nearly instantaneous physical combination of the reactants without a visible reaction. Some reactions stop here. There are tests available to detect these first-stage reactions; but, fortunately, many antigen-antibody reactions go to a second stage—a visible reaction. Although the manifestations of a visible reaction may take minutes or even hours to become evident, the serologic tests presently in use make wide use of second-stage reactions. In fact, many test reactions, that normally would not be visible, have been modified to provide visible reactions for the serologist.

c. Types of in vitro Antigen-Antibody Reactions. There are several types of antigen-antibody reactions used by the serologist in his profession. Although there are many modifications of the basic reactions, they can be classified into the following five main categories: precipitation, agglutination, neutralization, complement fixation, and immunofluorescence reactions or tests. Since a given antibody may react in many different ways, there may be tests available from several of these categories for a given disease. For example, a disease may stimulate the production of IgM antibodies which are most efficient in agglutination reactions, but these antibodies will react in other types of reactions too. Consequently, test selection must be based on the antibody present as well as on the equipment and personnel capabilities of a given laboratory.

2–8. Precipitation Reactions. Precipitation is the second-stage reaction in which the antibody (precipitin) reacts with a soluble antigen. The visible precipitate results from the sequential combination of antibodies and antigens until antigen-antibody aggregates large enough to be seen are formed. This formation of visible aggregates will occur only when optimum proportions of both antigen and antibody are present. Most precipitation reactions take several hours for a visible precipitate to appear. The tests are usually performed by one of two basic methods—in capillary tubes or by immunodiffusion in agar.

a. Capillary-Tube Precipitin Tests. In this technique, the soluble antigen and the antibodies (antisera) are introduced successively by capillary attraction into the tube so that the layers contact each other. After incubation in an upright position, the precipitate usually forms at the antigen-antibody interface or throughout the mixture if the proper antigen and antibody are present. This test is relatively insensitive as it detects only large amounts of antibodies. Another more obvious limitation of this method is that both antigen and antibody solutions must be clear, as cloudiness may interfere with reading the final results. This technique is used for the detection of C-reactive protein in the serum. Precipitin tests also have applications in bacteriology in the classification of streptococci.

b. Immunodiffusion Tests. There are many tests being developed which depend on the diffusion of either the antigen or antibody (or both) through a buffered agar before they react. Some of these methods require sophisticated laboratory equipment, such as for the detection of radioisotopes, and are beyond the scope of this manual, but the increased sensitivity and specificity of these methods may soon make them practical. However, there are a few immunodiffusion tests which rely on the formation of a visible precipitate. Some of these are rather sophisticated tests too, but their importance warrants at least brief mention. In general, these tests are based on the migration of the antigen and/or antibody through the agar until optimum proportions of each are reacted and a precipitate appears. As usual, the appearance of the precipitate takes several hours, but a modification by combining diffusion with electrophoresis has speeded the reaction and has great promise for the future in serologic diagnosis.

(1) Double Diffusion Tests. In double diffusion tests, the antigen and antisera are placed in separate wells cut into the agar and each diffuses toward the other. A precipitate line appears where optimum proportions of the reactants are present. See figure 2–3. Although the technique yields only qualitative results, it is simple and can be used with mixed antigen systems or in many combinations of known-unknown situations. The test is referred to as the Ouchterlony test. It has been applied to the detection of antibodies in coccidioidomycosis.

(2) Single Radial Diffusion Tests. In this method, one reactant, usually the antibody, is incorporated into the agar. The antigen is placed in a well and diffuses radially into the agar. A ring of precipitate forms whose diameter is proportional to the antigen concentration (see figure 2–4). Standard antigens of known concentrations may be tested at the same time. The diameter of the reactions of the standards can be plotted against their concentration; and, then, the concentration of unknown samples can be read directly from the standardization curve. This method is presently used for estimating the concentration of immunoglobulins (IgG, IgM, etc) and complement (C'3). The specific details of these tests are described in chapter 15. Several other antigenic substances can also be assayed by this method.

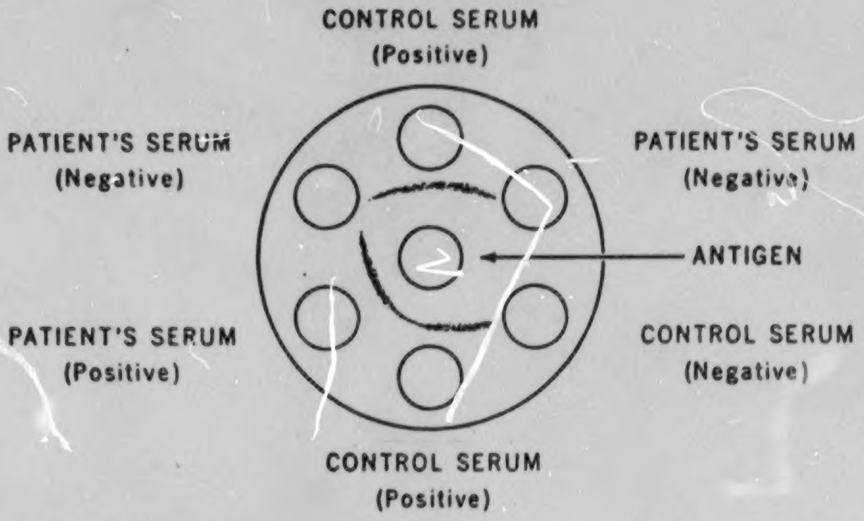

CONTROL SERUM
(Positive)
PATIENT'S SERUM
(Negative)
PATIENT'S SERUM
(Negative)
ANTIGEN
PATIENT'S SERUM
(Positive)
CONTROL SERUM
(Negative)
CONTROL SERUM
(Positive)

Figure 2-3. Double Diffusion (Ouchterlony).

(3) Immunoelectroosmophoresis (IEOP). This technique is also called counterelectrophoresis, counter-current electrophoresis, counterimmunoelectrophoresis and others. Basically the principle is a combination of simultaneous diffusion and electrophoresis followed by precipitation at the point of optimal proportions of reactants. In the test, antigen and antibody are placed in separate wells in the agar and an electrical current applied. The pH and ionic strength of the agar as well as the nature of the antigen and antibody determine the migration characteristics of the system. As applied to the detection of the hepatitis associated antigen (HAA) in the blood of potential blood donors, the antigen (HAA) is electrophoresed toward the known antibodies which are diffusing through the agar toward the antigen. A precipitate line appears when optimum proportions of each have reacted (see figure 2-5). The main advantages of IEOP over double diffusion tests are increased speed and the ability to detect very small quantities of antigen—both important considerations for the clinical serologist.

2-9. Agglutination Reactions. These reactions are similar to precipitation reactions in that antigen-antibody aggregates are formed. The main difference is that agglutination is used to describe the aggregation of particulate antigens. These antigens might be red blood cells, bacteria, or even inert particles such as latex particles that have been coated with a given antigen. Flocculation is at times used erroneously as a synonym for agglutination. Some authors use agglutination when describing reactions involving cellular antigens (red blood cells, etc) and flocculation for inert antigens (latex, etc). Many varieties of agglutination (or flocculation) reactions have been applied to clinical serol-

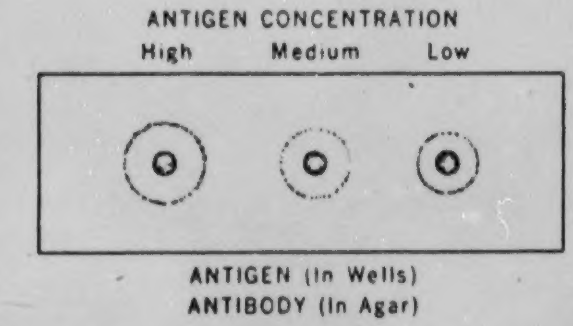

ANTIGEN CONCENTRATION
High
Medium
Low
ANTIGEN (In Wells)
ANTIBODY (In Agar)

Figure 2-4. Single Radial Diffusion.

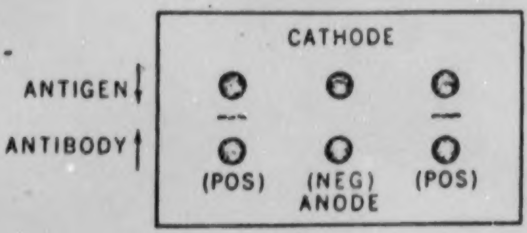

CATHODE
ANTIGEN
ANTIBODY
(POS)
(NEG)
(POS)
ANODE

Figure 2-5. Immunoelectroosmophoresis (IEOP), Example Using Australia Antigen.

ogy. They are faster than precipitin reactions and many can be observed macroscopically.

a. Bacterial Agglutination. The first practical application of serologic test in medicine was for the detection of agglutinating antibodies (agglutinins) against *Salmonella typhi* in the serum of patients with typhoid fever. Although this test was devised before the turn of the century, it is still in use and the principle has been applied to the serodiagnosis of certain other bacterial and rickettsial diseases such as tularemia, brucellosis, and typhus. In these tests, known bacterial antigens are used to detect unknown antibodies in the patient. The bacteria may be live or killed and preserved in some fashion such as with heat and/or formalin. In the case of typhus and related rickettsial diseases, a good example of the application of cross-reactivity is seen. Agglutination tests for rickettsial diseases use bacteria of the genus *Proteus* as antigens instead of the rickettsiae that actually cause the disease. The febrile agglutination tests in chapter 5 are bacterial agglutination tests. Another important application of bacterial agglutination is in bacteriology, where known antisera (antibodies) are used to identify unknown bacteria.

b. Hemagglutination. The agglutination of erythrocytes by antibodies or viruses provides the basis for many serotests. In addition, hemagglutination reactions are the main type used in immunohematology or blood banking but this field is covered in another manual. There are several modifications of the basic test. Each will be discussed separately.

(1) Direct Hemagglutination. In these tests the antigens on the erythrocytes are present normally. Although the cells may be preserved to increase their useful lifespan, they are essentially unaltered. Direct hemagglutination serotests are applied mainly to two diseases, primary atypical pneumonia and infectious mononucleosis. Both are excellent examples of the application of cross-reactivity to serotests. The antibodies in primary atypical pneumonia agglutinate human group "O" or the patient's own erythrocytes although the disease is caused primarily by a pleuropneumonia-like organism. In infectious mononucleosis, erythrocytes from sheep, horses, and oxen have been used to detect antibodies. Tests for infectious mononucleosis and primary atypical penumonia are found in chapters 7 and 8, respectively.

(2) Passive (Indirect) Hemagglutination. The antigens in this type of hemagglutination test have been adsorbed onto erythrocytes. The erythrocytes are simply physically carrying the antigens. Sheep erythrocytes are frequently used for this purpose. A wide

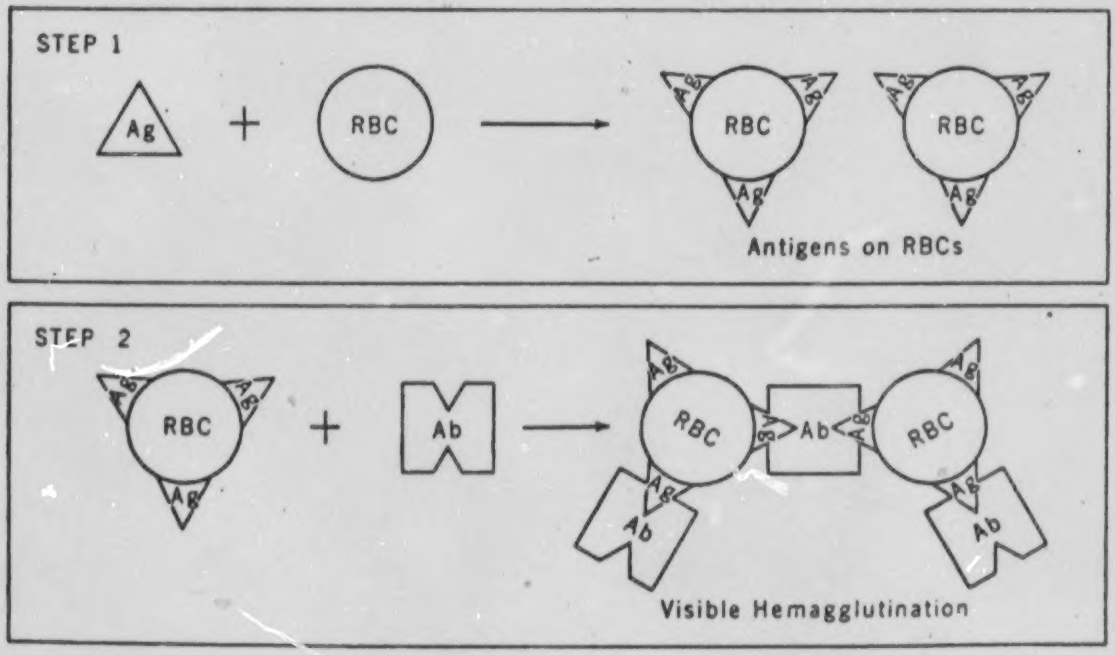


Figure 2-6. Passive (Indirect) Hemagglutination.

variety of antigens which do not normally give a second-stage reaction of any kind can be detected if the antigens are adsorbed on erythrocytes. Agglutination of the erythrocytes is indicative of the antigen-antibody reaction. Many polysaccharide antigens will adsorb directly onto erythrocytes but protein antigens usually require pretreatment of the erythrocytes with tannic acid before the antigens will adhere. Antigens prepared in this way provide a very sensitive method of testing for antibodies. The technique has been used in the diagnosis of amebiasis, plague, Hashimoto's thyroiditis, and others. The reaction is presented graphically in figure 2-6.

(3) Passive Hemagglutination Inhibition. Another useful application of antigens artifically adsorbed onto erythrocytes has been in tests to inhibit hemagglutination that would take place in the presence of the proper antibody. The test is used mainly to detect the presence of free antigens in solutions such as urine and serum. It is carried out in two steps. First, a known antibody against the antigenic substances under study is mixed with the solution that is suspected of

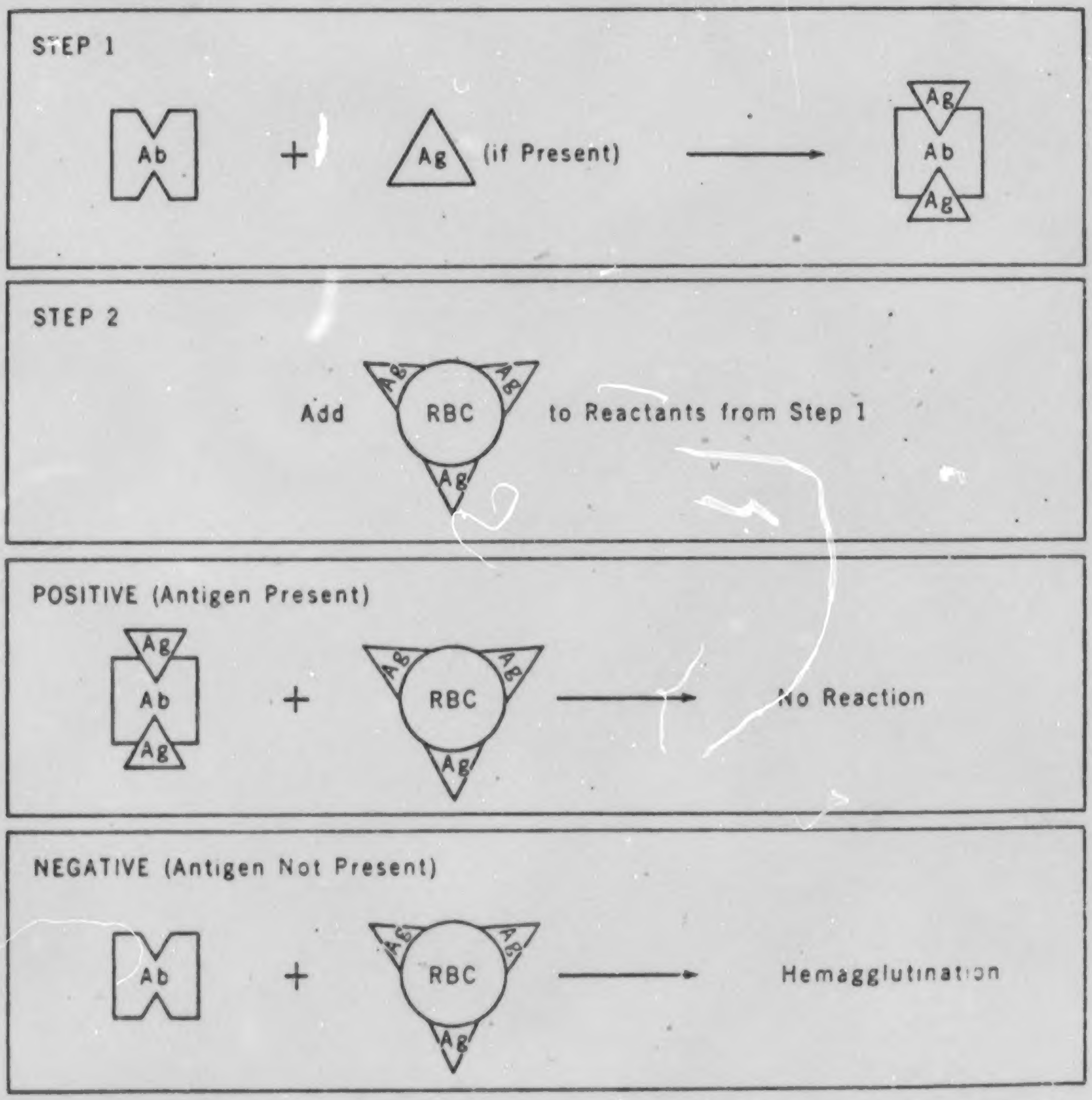

STEP 1
Ab
+
Ag
(if Present)
Ag
Ab
Ag
STEP 2
Add
Ag
RBC
Ag
Ag
to Reactants from Step 1
POSITIVE (Antigen Present)
Ag
Ab
Ag
+
RBC
No Reaction
NEGATIVE (Antigen Not Present)
Ab
+
RBC
Hemagglutination

Figure 2-7. Passive Hemagglutination Inhibition for Detecting an Antigen.

containing the antigen. If the antigen is, in fact, present, the antibody will be bound or inhibited. In the second step of the test, antigen-coated erythrocytes are added to the test. Agglutination now indicates that the antibody has not been inhibited and is free to react with the antigen-coated erythrocytes—a negative test. It means that the antigen was not present in the solution under study. No agglutination of the erythrocytes indicates a positive test. These reactions are presented graphically in figure 2-7. This technique has been applied successfully in pregnancy tests (see chapter 13) for the detection of human chorionic gonadotropin (HCG) in the urine.

(4) Viral Hemagglutination Inhibition. Certain viruses have the ability to agglutinate certain erythrocytes. For example, the rubella (German measles) virus agglutinates erythrocytes from day-old chicks. Antibodies against the virus inhibit this agglutination. The reaction is presented graphically in figure 2—8. Determining the titer of rubella

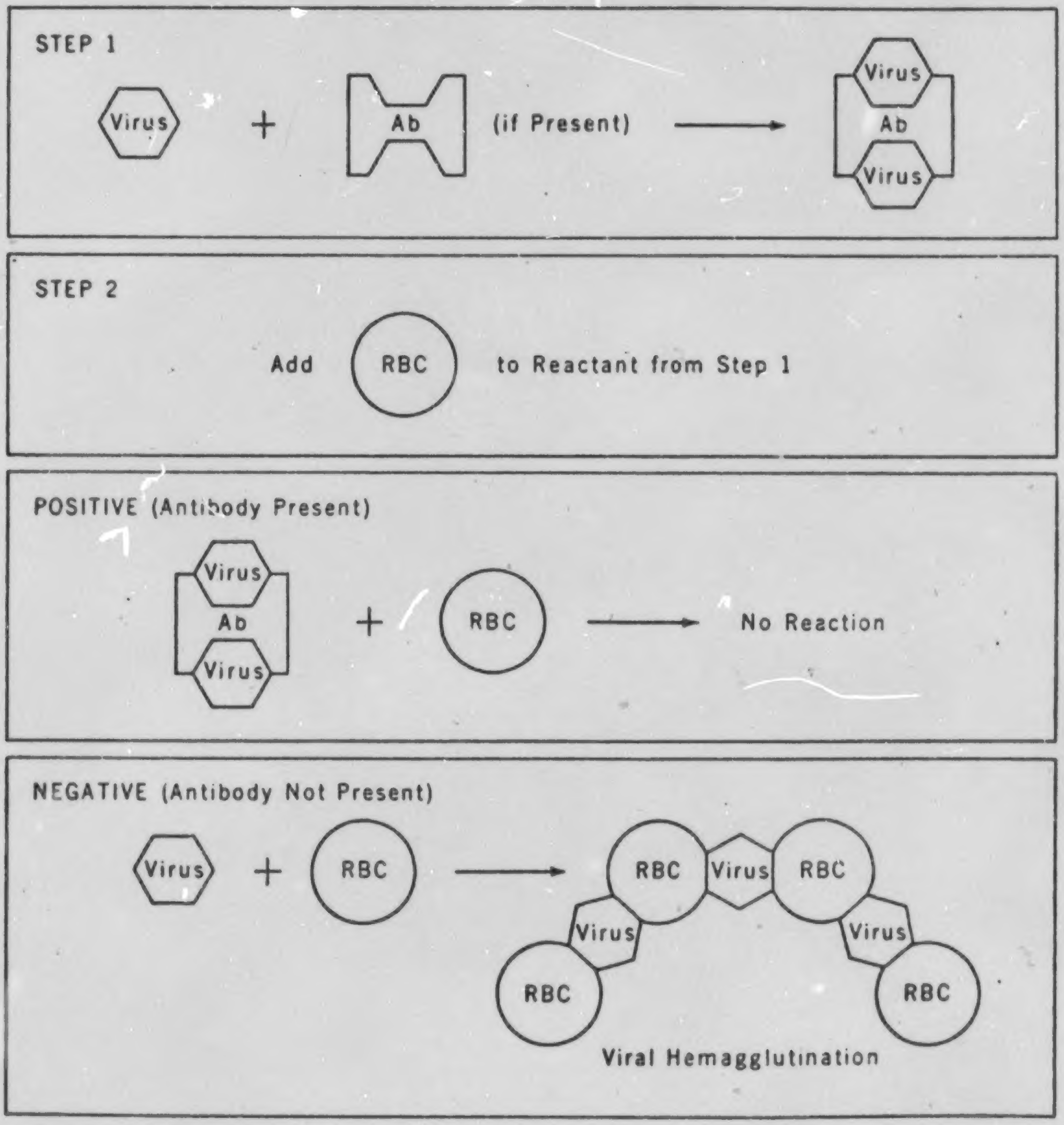

STEP 1
Virus
+
Ab
(if Present)
Virus
Ab
Virus
STEP 2
Add
RBC
to Reactant from Step 1
POSITIVE (Antibody Present)
Virus
Ab
Virus
+
RBC
No Reaction
NEGATIVE (Antibody Not Present)
Virus
+
RBC
RBC
Virus
RBC
Virus
Virus
RBC
RBC
Viral Hemagglutination

Figure 2–8. Viral Hemagglutination Inhibition for Detecting Antibodies.

hemagglutination-inhibiting antibodies in a patient's serum is a useful measure of the patient's ability to resist future infection by the rubella virus. This test for inhibiting antibodies has been used extensively on pregnant women where a rubella infection could result in severe damage to the developing fetus. Tests for rubella antibodies are in chapter 14. The same principle has been applied to the serodiagnosis of influenza and certain arbovirus diseases which also result in the production of hemagglutination-inhibiting antibodies.

(5) Agglutination Involving Inert Particles. Several inert, acellular particles have been coated with either antigen or antibody and used in serotests. Some of these are latex, bentonite, charcoal, and others. The reactions involved may be either direct agglutination of the coated particles or agglutination inhibition. Too many diverse combinations of antigens and inert particles exist to cover adequately at this point so each will be covered in this manual as they apply to each test, but tests for syphilis and rheumatoid arthritis are examples.

2–10. Neutralization Reactions. Usually neutralization is used only to describe antigen-antibody reactions which require a laboratory animal to detect whether or not a reaction has taken place. The antibodies and antigens, such as toxins or viruses, are mixed in vitro. Then the mixture is injected into the test animal. Subsequent observation of the animal for characteristic symptoms indicates whether the toxin or virus has been neutralized. As can be imagined, the maintenance of an animal colony is beyond the scope of the average clinical laboratory. However, if one uses the term, neutralization, in a broader sense, there are several serologic tests that could be categorized as neutralization reactions. For example, the hemagglutination inhibition tests previously discussed might apply, but the best example is the antistreptolysin-O (ASO) test. The antigen in this test is a hemolysin, streptolysin-O, that is produced by certain streptococci. Antibodies neutralize the hemolysin in vitro so that when erythrocytes are eventually added to the test, the hemolysin can no longer hemolyze them. This test is presented in chapter 9.

2–11. Complement Fixation Reactions. Complement is a set of 11 serum constituents which become inactivated (fixed) in many antigen-antibody reactions. In fact, complement is required for the completion of many of these reactions. However, since many antigen-antibody reactions do not result in a visible manifestation, the detection of complement fixation provides a useful serologic test. To detect whether complement fixation has occurred in the first reaction, a second antigen-antibody system is added to the test. Sheep erythrocytes and antisheep erythrocyte antibodies (hemolysins) are usually used in this second step. The sheep RBC hemolysins require the presence of complement to lyse the erythrocytes. Therefore, lysis of the erythrocytes indicates that free complement is still present in the system or that no complement fixation (no antigen-antibody reaction) occurred in the first step of the test. Conversely, no hemolysis indicates a positive complement fixation test. This test has many applications in the serodiagnosis of viral diseases and has also been used extensively in syphilis serology. However, since the test is performed only in larger laboratories, procedures will not be included in this manual and reference should be made to procedures in the medical literature when needed.

2–12. Immunofluorescence Reactions. Also called fluorescent antibody (FA) reactions, these tests are based on the fact that certain chemicals emit visible light when exposed to ultraviolet (UV) light. Some of these chemicals (for example, fluorescein isothiocyanate) can be conjugated with antibodies without destroying antibody reactivity. Although there are several modifications, two main types of reactions are used in the fluorescent antibody tests in use in clinical serology.

a. Direct Fluorescent Antibody Technique. In this method, a specific antibody is conjugated with fluorescein. The labeled antibody is reacted on a slide with its specific antigen, such as bacteria, spirochetes, viruses, or similar particulate antigens. The specific antibodies adhere to the cells. After washing away excess antibody (and any other antibodies not specific for the antigen), the preparation is exposed to UV light and viewed with a microscope fitted with special filters. In the case of fluorescein isothiocyanate-labeled antibodies, the organisms will now emit a blue-green light. This technique is used mainly to detect an antigen in tissue or from culture by using known, labeled antibodies. It has been applied to the detection of the gonococcus, meningococcus, streptococci, plague bacilli, and several others in clinical specimens.

b. Indirect Fluorescent Antibody Technique. This modification of the fluorescent antibody technique is useful because it permits the detection of antibody in a patient's serum without the time-consuming process of having to label the antibodies of each patient with the fluorescein dye. As usually applied, the unlabeled patient's serum is reacted with a known antigen (bacteria, spirochetes, etc.) on a slide and the excess antibody washed away as in the direct technique. Next, fluorescein-labeled antihuman globulin, that has been produced in an animal following the injection of human globulin is added. The labeled antihuman globulin reacts with the patient's antibodies (globulins) that have coated the antigens. When examined by UV microscopy, the same fluorescence as in the direct test is seen. The steps in the reaction are diagrammed in figure 2-9. In addition, this test can be performed on serial dilutions of the patient's serum in order to determine the antibody titer. The Fluorescent Treponemal Antibody Absorption (FTA-ABS) test for syphilis in chapter 6 and the Antinuclear Antibody Test for lupus erythematosus in chapter 12 are based on this indirect FA method.

2-13. **Other Antigen-Antibody Reactions.** Several other types of reactions occur which require at least brief mention. Some of these could have been included in the five categories described in the preceding paragraphs but are considered to be of lesser importance than the examples given. Others are tests of the future. A few of these will be described briefly.

a. **Opsonification.** This process is the alteration of foreign particles by antibodies (opsonins) which enhance phagocytosis of the particles. In vitro tests are available to detect these opsonins.

b. **Quellung Reaction.** Antibodies against certain encapsulated bacteria, such as pneumococci, cause the capsules to swell. The reactions are very specific. In the past these reactions were used extensively in selecting the proper antiserum to administer to pneumonia patients, but their use has nearly disappeared since the advent of antibiotics. This capsular swelling by specific antibody has also been used in the identification of *Haemophilus influenzae* and *Bacillus anthracis.*

c. **Immunoelectrophoresis (IEP).** Electrophoresis of serum antibodies in agar may be followed by the addition of known precipitating antiglobulins, such as anti-IgM, anti-IgG, etc. When the amount of precipitate is compared with known normal standards, a semiquantitation of each class of immunoglobulin is possible. Through the use of radioisotopes, many modifications of the basic technique are possible.

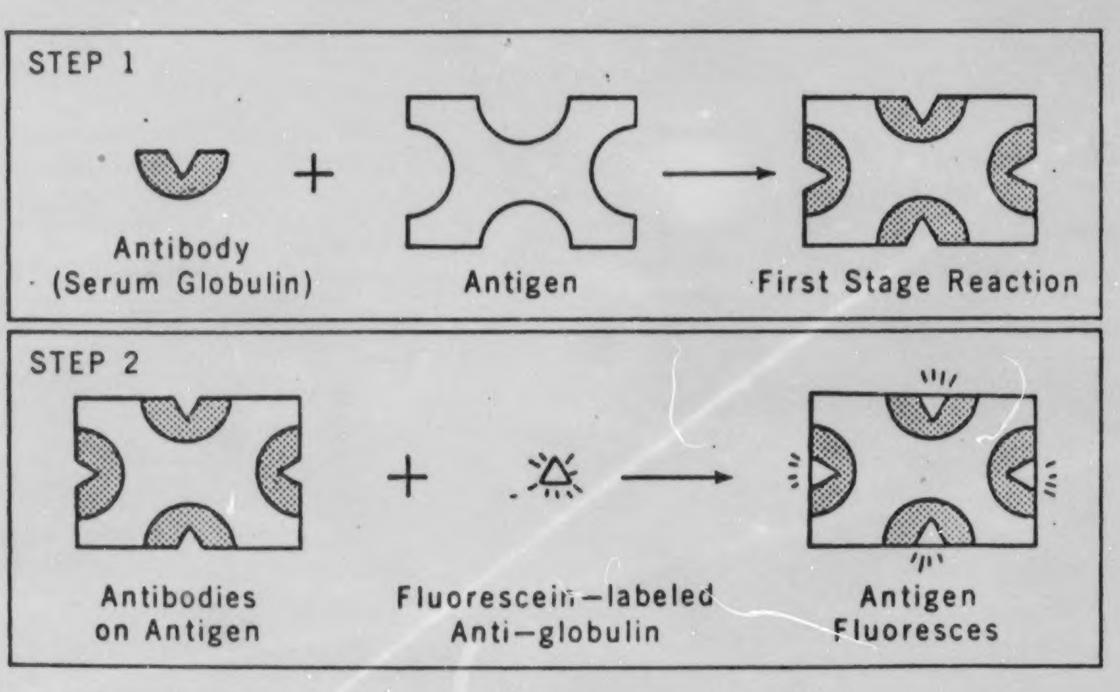


Figure 2-9. Indirect Fluorescent Antibody (Immunofluorescence).

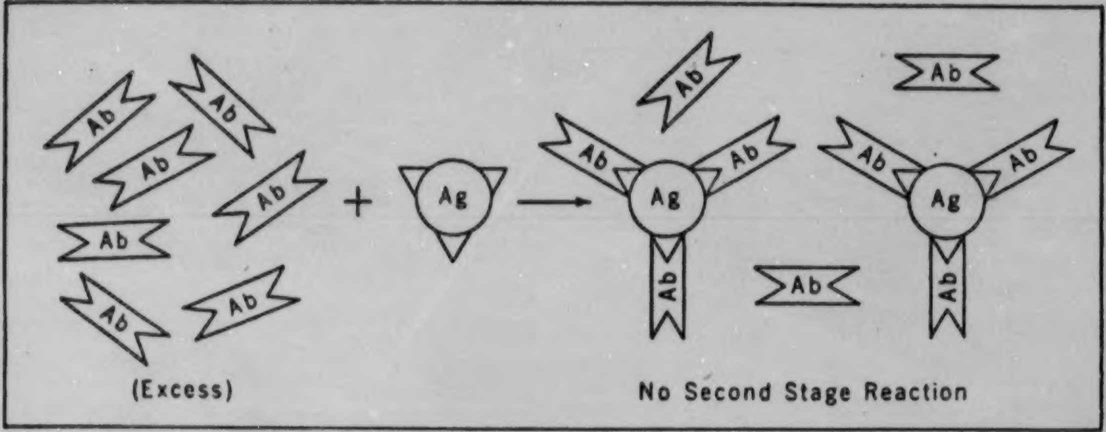


Figure 2–10. Prozone (Excess Antibody) Reaction.

d. **Radioimmunoassay (RIA).** This term applies to serologic tests which employ radioisotopes in them. Although limited at present to larger laboratories, these tests are likely to become more widely used due to their increased sensitivity and greater accuracy than other methods. Presently under development is a test for the hepatitis associated antigen, that is several times more sensitive than older methods.

2–14. ZONAL REACTIONS. There are several factors, such as ionic concentration, temperature, incubation time, and others, that affect antigen-antibody reactions. However, the serologist rarely has to worry about these factors because most reagents used in serology are commercially manufactured. All the serologist has to do is follow the manufacturer's directions with adequate controls, and these factors will be kept under control. However, there are two situations of which the serologist should be aware. Both may result in a weak reaction or lack of visible manifestation of the expected reaction due to alterations in the optimal proportions of antigen to antibody.

a. **Prozone Reactions.** These are weak or negative reactions due to an antibody excess. For example, in an agglutination reaction, each antigenic determinant might be combined with a single antibody molecule so that agglutination would be impossible. A prozone reaction is depicted graphically in figure 2-10. Suspected prozone reactions can be detected by making dilutions of the antibodies (serum) before adding the antigen.

b. **Postzone Reactions.** These are weak or negative reactions due to an excess of antigen in the test. Although rarely encountered by the

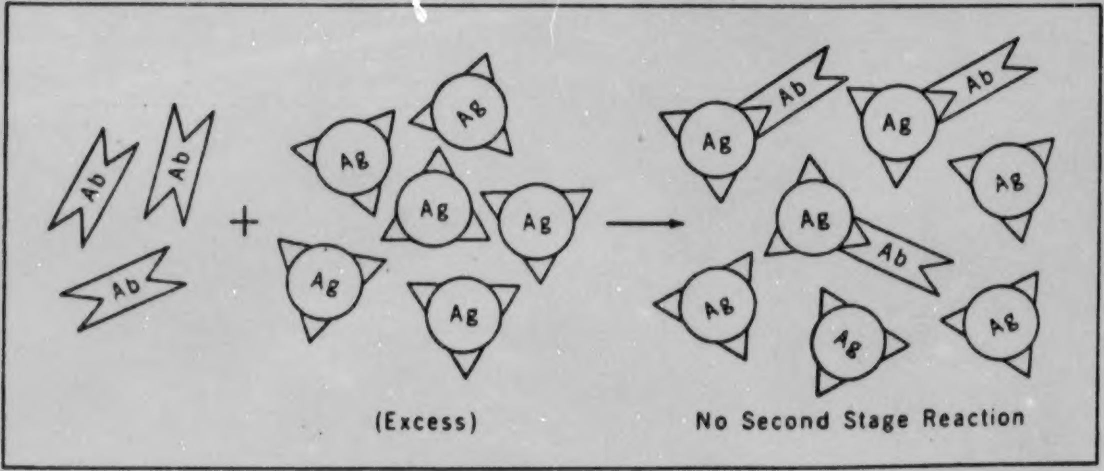


Figure 2–11. Postzone (Excess Antigen) Reaction.

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serologist unless he is devising or modifying a test, postzone reactions are, in fact, what are seen early in a disease before an optimal antibody level is achieved. Some undetectable antibody may be present. A postzone reaction is depicted graphically in figure 2-11.

Chapter 3

SPECIMEN COLLECTION AND PREPARATION

3-1. Types of Specimens. Valid serologic tests must be performed on properly collected and prepared specimens. Since most serotests are performed on the patient's serum, proper collection and preparation usually refers to blood collection and separation of serum. This chapter is devoted primarily to the processing of serum. However, certain serotests are performed on other body fluids. In addition to serum, some serotests are performed on plasma, cerebrospinal fluid (CSF), and urine. The characteristics of each type of specimen will be described briefly.

a. **Serum.** Serum is the supernatant portion of whole blood that has been allowed to clot. Serum is the most frequently assayed specimen in serology because antibodies are found in the serum. Certain abnormal components, such as C-reactive protein (CRP) and rheumatoid factors, are also found in serum by using serotests.

b. **Plasma.** Plasma is the fluid portion of blood that has been prevented from clotting by the addition of anticoagulants, such as oxalates, heparin, EDTA, and others. The only significant difference between plasma and serum is that (in addition to anticoagulants) plasma contains the fibrinogen that is removed during the clotting process. Plasma is rarely used in serotests because fibrinogen precipitates when plasma is heated, and anticoagulants could alter the sensitive chemical balance of a serotest. However, certain tests for syphilis may use unheated plasma and unheated serum interchangeably. Therefore, be sure to use plasma only if specified in a given test procedure and then only if the correct anticoagulant is used.

c. **Cerebrospinal Fluid (CSF).** A physician collects this specimen and usually presents the laboratory with three tubes of fluid numbered in the order collected. The third tube is usually reserved for serotests, but any blood-free sample could be used. Serotests on CSF are usually limited to tests for syphilis, but tests are available for detecting albumin and immunoglobulins in CSF.

(1) Cerebrospinal fluid is also frequently tested for its effect on various colloidal suspensions, such as colloidal gold, gum mastic, and benzoin. Although colloidal tests are not really serologic tests, some serologic techniques are employed in performing them so the serologist is usually responsible.

(2) One very important precaution to keep in mind when handling CSF is that, like any other biological fluid, CSF, must be considered to contain pathogenic microorganisms until proven to the contrary. The specimen may have been collected from a patient with meningococcal meningitis, tuberculosis, or some other infectious disease transmittable to the unwary technician.

d. **Urine.** Pregnancy tests are about the only serotests performed on urine in the average clinical laboratory. These are tests for the presence of a hormone, human chorionic gonadotropin (HCG), in the urine during pregnancy. Usually a freshly collected morning specimen will be used in these tests. In addition to pregnancy, HCG, may be detected in the urine in certain diseases. Urine is also occasionally tested for the presence of certain immunoglobulins that may be passed in abnormal conditions, but these tests are usually beyond the scope of the average clinical laboratory.

3-2. Blood Collection, Venipuncture. There are several reasons why serum from blood collected by venipuncture is the specimen of choice for many serologic tests. Primarily, if the antecubital region of the forearm is used as a collection site, the patient's discomfort is minimal and of short duration. In addition, the venipuncture is easily performed by a technician, and enough blood can be collected at one time for several tests or repeat tests. The techniques to be described here are applicable to all serotests requiring serum or plasma except for the tests for cold agglutinins. The special techniques for collecting serum for cold agglutinins are covered in chapter 8.

a. **Venipuncture Site.** Venous blood can be collected from several body sites. A physician should make all collections from the scalp, femoral, or jugular veins. However, even minimally trained technicians should be able to collect blood from the antecubital region of the arm of most patients. If necessary, the technician may also collect blood from the back of the hand or top of the foot. However, the back of the hand and foot are more sensitive to pain, and the veins tend to roll easily so that the arm is usually selected as the site of choice. The venous system of the antecubital region of the arm is shown diagrammatically in figure 3-1. The actual collection site is usually the medium cubital vein or another vein in that vicinity.

b. Equipment. In general, sterile equipment or glassware should be used in performing venipunctures for serotests. The main items used in venipunctures are as follows:

(1) Syringes or vacuum blood-collecting tubes (usually without anticoagulant).

(2) Needles, sterile, disposable, about 20 gauge, 1½ inches long (or double-pointed needles).

(3) Tourniquet.

(4) Isopropyl alcohol, 70%. If made from 99% isopropyl alcohol, q.s. 70 ml of alcohol to 99 ml with distilled water.

(5) Alcohol-soaked sponges. Use 70% alcohol.

(6) Gauze pads, sterile, 2 × 2 inches.

(7) Labeled collecting tubes.

c. Preparatory Steps:

(1) Label the collecting tubes with the patient's name and other identifying information.

(2) Wash your hands thoroughly.

(3) Assemble the needle and syringe or vacuum tube system. Align the bevel with the markings on the syringe. Examine the needle and discard damaged ones. Keep the plastic shield on the needle until ready to use. Make sure that the plunger of the syringe is free and pushed completely into the barrel to prevent injecting air during the venipuncture.

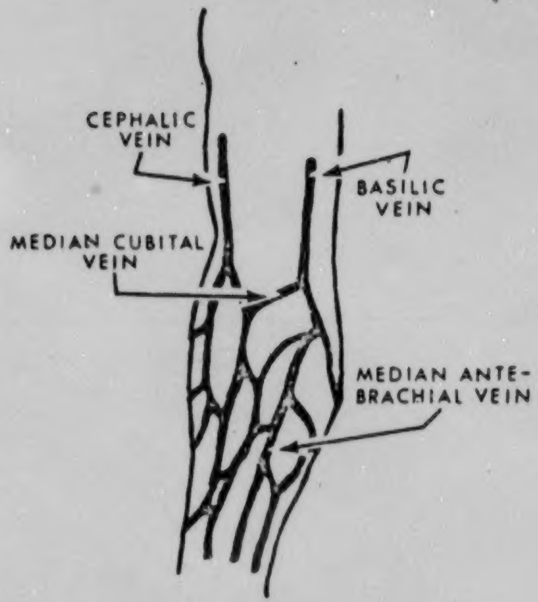


Figure 3-1. Site of Venipuncture.

(4) If ambulatory, have the patient seated with the selected arm extended plam upward and resting on a table or similar device. Bed patients may rest their arms on the bed.

d. Syringe Technique:

(1) Place the tourniquet about 3 inches above the patient's elbow just tight enough to check venous flow but not arterial flow. The patient should still have a pulse at the wrist.

(2) Have the patient open and close the fist several times.

(3) Select the desired vein by inspection and palpation (see a, figure 3-2).

(4) Clean the skin over the selected puncture site with an alcohol-soaked sponge (see b, figure 3-2). Wipe off excess alcohol with a dry, sterile gauze pad. Maintain aseptic technique from this point on.

(5) Have the patient clench his fist.

(6) Grasp the syringe in the right (or left) hand and remove the plastic shield from the needle. The forefinger may be placed on the hub of the needle as a guide. Do not touch the needle. With the other hand, hold the skin taut about 2 inches below the puncture site (see c, figure 3-2).

(7) With the needle held bevel up, insert the needle alongside and parallel to the vein for a short distance and then into the vein, all in one motion (see d, figure 3-2).

(8) Gently aspirate the desired quantity of blood. Make sure to firmly anchor the barrel of the syringe while aspirating (see e, figure 3-2).

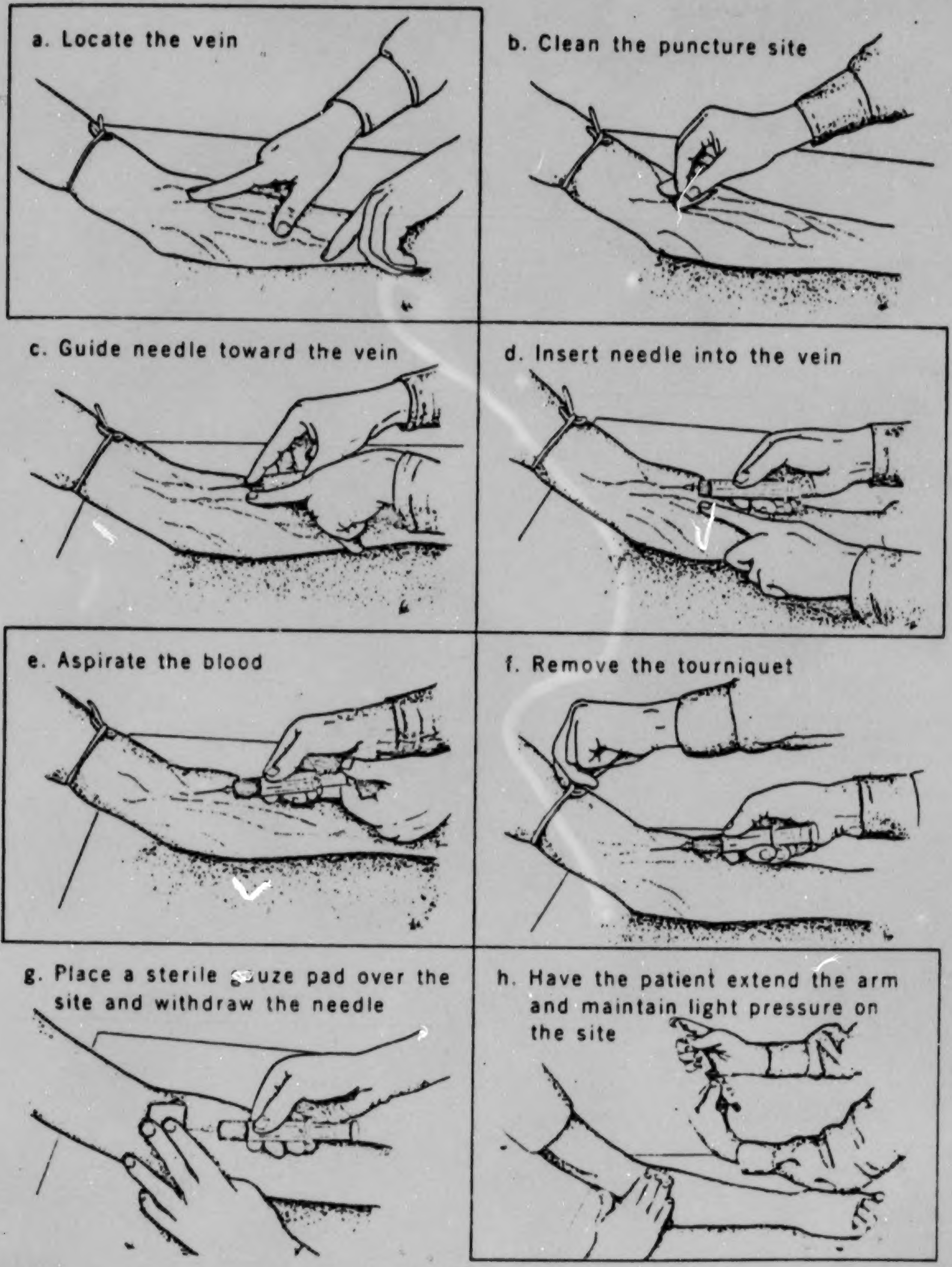
(9) Remove the tourniquet prior to removing the needle (see f, figure 3-2) and have the patient relax his fist.

(10) Place a dry, sterile gauze pad over the puncture site and apply light pressure. Withdraw the needle in one smooth motion (see g, figure 3-2).

(11) Have the patient keep constant pressure on the site for 3-5 minutes while keeping his arm extended. Remove the needle from the syringe and gently deliver the blood specimen down the side of the appropriately labeled tube (see h, figure 3-2).

e. Vacuum Blood Collecting Tube Technique:

(1) Label the vacuum tube with the pa-



a. Locate the vein

b. Clean the puncture site

c. Guide needle toward the vein

d. Insert needle into the vein

e. Aspirate the blood

f. Remove the tourniquet

g. Place a sterile gauze pad over the site and withdraw the needle

h. Have the patient extend the arm and maintain light pressure on the site

Figure 3-2. Venipuncture Procedure.

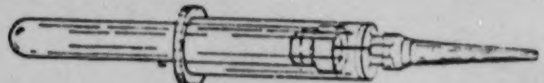


Figure 3-3. Vacuum Blood Collection System.

tient's name and other identifying information.

(2) Screw the double-pointed needle into the holder, leaving the plastic shield over the venipuncture point.

(3) Embed the short needle in the stopper until the stopper reaches the guideline on the holder. Do not break the vacuum (see figure 3-3).

(4) Prepare the patient and insert the needle as indicated in paragraph d.

(5) Once in the vein, push the tube all the way into the holder. Constant pressure on the end of the tube may be required if the needle is equipped with the rubber shut-off sleeve for collecting multiple specimens. The sleeve tends to push the tube from the short needle.

(6) Change tubes if multiple tubes are to be collected.

(7) Remove the tourniquet, apply a sterile gauze pad and remove the needle as described in paragraph d. Be sure that the patient maintains constant pressure on the site for 3-5 minutes following the venipuncture.

f. Discussion:

(1) Aseptic technique is required for the patient's and technician's safety.

(2) If possible, collect blood at least an hour after the patient has eaten, since chylous serum could interfere with certain serologic tests.

(3) To minimize the development of a hematoma, be sure to remove the tourniquet before withdrawing the needle or if difficulty is encountered in inserting the needle.

(4) Blood collected in syringes must be transferred quickly and gently to the specimen container. Hemolyzed blood is unacceptable for serologic tests.

(5) Always allow vacuum tubes to fill completely because hemolysis may occur due to rushing air as the needle is withdrawn.

(6) The blood-collecting techniques described in this chapter are applicable to all serotests requiring serum except for cold hemagglutinin tests. Special collection techniques for cold agglutinins are given in chapter 8.

3-3. Blood Collection, Capillary Puncture. Blood collection from capillary punctures is seldom required for serologic tests because venipuncture is a relatively simple and efficient technique. However, capillary puncture may prove to be the most practical method with certain patients, such as infants.

a. Puncture Site:

(1) Adults. Usually the lateral surface of the tip of the ring or middle finger is used when the patient is an adult. Heavily calloused areas should be avoided. As an alternate site the edge of the ear lobe is acceptable.

(2) Infants. Either the heel or the big toe can be used for collecting blood by capillary puncture from infants.

b. Reagents and Equipment:

(1) Isopropyl alcohol, 70%. If made from 99% isopropyl alcohol, q.s. 70 ml of alcohol to 99 ml with distilled water.

(2) Alcohol-soaked sponges. Use 70% alcohol.

(3) Gauze pads, sterile, 2 × 2 inches.

(4) Lancets, finger-bleeding, sterile.

(5) Capillary tubes, with or without heparin as determined by the test procedure.

c. Technique:

(1) Be sure the selected puncture site is warm and has adequate circulation. The site may be warmed with warm [illegible]C–40°C) water for a few minutes. Dry the site before the next step.

(2) Clean the site with an alcohol-soaked sponge. Wipe the site dry with a dry, sterile gauze pad. Be sure the site is completely dry to prevent hemolysis of the blood by alcohol.

(3) Make the puncture and wipe away the first drop of blood. NOTE: An "X" incision is advised for small infants to assure a freely flowing sample.

(4) Fill the capillary tubes about three-fourths full by capillary attraction.

(5) Apply pressure to the wound with a sterile gauze pad (or have the patient apply pressure) until bleeding stops.

3-4. Separation of Serum:

a. Clotting. Blood for all serotests, except cold agglutinins (see chapter 8), can be allowed to clot at room temperature. A few minutes after the blood is collected, free the developing clot from the tube by ringing the clot with an applicator stick. Usually enough serum for most tests will be expressed after about 30 minutes to an hour. If greater quantities of serum are required and the delay is not significant, the clot may be allowed to retract more completely overnight in the refrigerator (4°C–10°C). When adequate serum has been expressed from the clot, the clot is sedimented by centrifugation.

b. **Centrifugation.** Depending on the amount of serum required for testing, centrifugation for as little as 5 minutes at 1500 rpm may be adequate. For more complete packing of the clot, increase the time to about 15 minutes. Actually neither the time nor the speed is critical as long as the glassware can withstand the speed and adequate sedimentation of the clot is achieved. Blood collected in capillary tubes is best separated using a microhematocrit centrifuge after first freeing the clot from the glass with a bacteriological needle and sealing one end.

c. **Separation.** If serotests are to be performed immediately after centrifugation and no further treatment of the serum is required before testing, the serum may be left on the clot. Just be sure not to disturb the clot when taking a sample of serum. However, never store serum with the clot. If prolonged storage or shipment of the serum is anticipated, transfer the serum to another appropriate container. Be sure that the new container is tightly stoppered to prevent contamination and desiccation. After centrifugation, serum may be collected from capillary tubes by first scoring the tube with a file and then breaking the tube above the cells.

3–5. Preservation of Serum. Ideally, serotests should be performed as soon as possible after the serum is collected. This is necessary because antibodies in the serum are readily denatured by many microbial contaminants. However, since immediate processing is not always practical (or even possible, if the specimen must be shipped to another laboratory), some method of preserving the serum must be used. Specifically, the antibodies must be preserved. Both physical and chemical preservation methods are available.

a. **Physical Preservation:**

(1) Refrigeration. The best short-term preservation method for serum (and the least likely to interfere with the test) is simple refrigeration at 4°C–10°C. If separated from the clot, serum in tightly stoppered containers may be stored at this temperature for 2–3 days or longer. Serum should not be stored on the clot longer than overnight. Since most serologic tests are temperature-sensitive, be sure to allow the serum to return to the specified temperature before testing. For example, serum for most tests for syphilis must be at room temperature when tested.

(2) Freezing. Serum can be kept nearly indefinitely if frozen at minus 20°C–70°C in tightly stoppered containers. The serum must be free of cells before it is frozen because they will lyse on thawing. In addition, serum for serotests to be shipped to other laboratories is preferably shipped frozen in dry ice. Care must be taken to thoroughly (but gently) mix the serum for testing once it has thawed because it tends to separate on thawing. Of course, the thawed serum must also be allowed to attain the appropriate temperature before testing.

(3) Lyophilization. Removing the water from serum by freeze-drying the serum under high vacuum is an effective way to preserve serum. Lyophilized serum can be stored in tightly stoppered containers for long periods of time, especially if refrigerated. In addition, lyophilized serum can be shipped without refrigeration. The serum is reconstituted by simply replacing the appropriate volume of water and gently mixing. Unfortunately, lyophilization requires powerful vacuum pumps and other specialized equipment, thus usually limiting the technique to larger laboratories. The technique is usually not practical on a small scale anyway.

b. **Chemical Preservation.** Normally, chemical preservatives should not be added to serum that is to be used for serotests. However, serum and cerebrospinal fluid for syphilis testing can be effectively preserved with sodium ethylmercurithio-salicylate (Merthiolate, Eli Lilly and Co.). Be sure that Merthiolate will not interfere with test results before this preservative is used for other tests. Preservation is only by bacteriostasis and does not prevent denaturation of antibodies by heat or other means. Dry, powered Merthiolate must be used. Do not use Tincture of Merthiolate. Tubes are prepared by adding 0.1 ml of 1% (w/v) aqueous Merthiolate. The tubes are desiccated over calcium chloride in a vacuum desiccator in the dark. The tubes are tightly stoppered and stored in the dark, preferably in the refrigerator where they may be kept for several months. This amount of Merthiolate inhibits most bacterial growth in 2–8 ml of serum or spinal fluid.

3–6. Inactivation of Serum Complement. As described in chapter 2, the fixation of complement in certain antigen-antibody reactions is a useful serologic test. However, the native complement in the patient's serum also interferes with several other serotests and must be inactivated before the test is performed. In fact, even in complement fixation tests, the patient's native complement must be inactivated and a known amount of complement added to the test. On the other hand, there are certain serologic tests that require the presence of native complement, so follow each test procedure carefully in this regard. If inactivation of the native complement is required for a given procedure, two basic inactivation methods are available. Complement may be inactivated by carefully applied heat before the test or by certain chemicals in the reagents used in the test.

a. Heat Inactivation of Complement. Serum complement may be inactivated by heating at 56°C for 30 minutes in a water bath or dry-heat block. The serum must be at room temperature before heating; it must also be well mixed if it was previously frozen. Be sure that the serum is cell-free because the cells will hemolyze at inactivation temperatures. After heat inactivation, the serum should normally be allowed to cool to room temperature before testing. However, since some complement activity is regained on standing, any serum that has stood at room or refrigerator temperatures for longer than 4 hours after inactivation should be reheated at 56°C for 10 minutes and then allowed to recool before testing. Heat inactivation of plasma complement is impractical because the fibrinogen precipitates on heating. This precipitate must be removed before the plasma can be tested.

b. Chemical Inactivation of Complement. Both serum and plasma complement can be inactivated efficiently by incorporating choline chloride into the test reagents. Since heating is not required, the fibrinogen will not precipitate from plasma nor is the extra time (30 minutes) for heat inactivation required. With choline chloride, the inactivation apparently occurs instantaneously. At the present time, choline chloride is used primarily in certain tests for syphilis, which use unheated serum or plasma.

Chapter 4

FUNDAMENTAL SEROLOGIC PROCEDURES

4–1. Preparation of Saline. Saline is one of the most frequently used reagents in serology. Basically, saline is an aqueous solution of sodium chloride (NaCl), but there are many different concentrations and modifications in use so the technician must be sure to use the saline specified for each given procedure. Some of the variations in saline are as minimal as one procedure requiring saline containing 0.85% NaCl while another procedure may specify 0.90%. Other variations are more pronounced. For example, certain tests require saline that contains NaCl plus buffers such as glycine or phosphates. Many of these buffered salines are prepared to very critical specifications and pH ranges. Fortunately, most test kits provide the appropriate buffered saline as part of the kit. The buffered saline in these kits may be in aqueous form and ready to use or the constituents may be in powder form and need to be reconstituted by the serologist. Although most types of saline are available through supply channels, two varieties of saline can be prepared economically in the laboratory because large quantities of these are used regularly.

a. Normal Saline, 0.90%. Saline containing 0.90% (w/v) NaCl is called normal or physiological saline. Normal and physiological are also used to describe 0.85% saline. Except for the small difference in the amount of NaCl in each, 0.90% and 0.85% are essentially identical and for practical purposes can be used interchangeably. Saline (0.9%) is available through normal supply channels but may be easily prepared in the laboratory.

(1) Drying the Sodium Chloride. Dry the NaCl (reagent grade) in a flat, open container in a dry-air oven at 170%C ± 10°C for about 1/2 hour. Do not overheat the NaCl. Drying at 110°C for about 3 hours is also acceptable. Weigh the desired amount as soon as the container can be safely handled.

(2) Preparing the Saline. Weigh accurately 0.90 g of NaCl for each 100 ml of saline desired. Dilute the NaCl to the appropriate volume with distilled or deionized (demineralized) water.

b. Phosphate Buffered Saline (PBS), pH 7.2 ± 0.1. This buffered saline is used in large quantities in the Fluorescent Treponemal Antibody-Absorption (FTA-ABS) test. The premixed chemicals for this PBS are available commercially in powdered form. The powdered mixture requires only a single weighing before reconstitution. However, this PBS can also be prepared economically in the laboratory from the basic ingredients.

(1) Drying the Salts. Dry NaCl, Na_2HPO_4 and KH_2PO_4 for about 2 hours at 100°C–110°C in a dry-air oven. Do not dry phosphates containing water of hydration. Dry only anhydrous phosphates and do not exceed 100°C with these.

(2) Weighing the Reagents. Weigh accurately the following reagents for each liter of PBS required.

NaCl	7.65g
Na_2HPO_4	0.724 g
KH_2PO_4	0.21 g

(3) Preparing the PBS. Dissolve the salts and dilute to the appropriate volume with fresh or recently boiled distilled or deionized (demineralized) water. Determine the pH of the PBS on a pH meter. Lots that do not fall within ± 0.1 of pH 7.2 should be discarded.

(4) Storage of PBS. Store the PBS in tightly stoppered polyethylene or high-quality glass container preferably in the refrigerator. Be sure to allow an aliquot to warm to room temperature before pipetting.

4–2. Preparing Red Blood Cell Suspensions. Several serotests use suspension of erythrocytes (RBCs) as antigens. Depending on the particular serotest, these cells may be from man or from animals, usually rabbits or sheep. In addition to a specific animal source for the erythrocytes, the cells for each serotest must be of a specific blood group and concentration—usually from 2% to 5% by volume. Too many cells (antigens) in the test could result in a weak reaction due to the postzone effect (antigen excess). A weak reaction could also be produced by too few cells being used—the prozone effect (antibody excess). Another important factor is the age of the RBCs. Therefore, the serologist must be able to prepare accurate concentrations of RBC suspensions from cells of the proper age and blood group as well as from the proper animal source.

a. Collecting the Blood. Depending on the animal source, either venous or cardiac puncture blood may be used for RBC suspensions for serotests. The blood may be either defibrinated or mixed with an anticoagulant but anticoagulants are easier to use. If the cells are to be stored for significant periods of time, a preservative anticoagulant, such as ACD or Alsever's should be used.

(1) Acid-Citrate-Dextrose (ACD). Dissolve 1 g sodium citrate (monohydrated) and 1.5 g dextrose in 60 ml of water. Mix 1 part of ACD with 4 parts of blood. Blood collected in ACD will keep for several weeks in the refrigerator. Vacuum blood-collecting tubes containing ACD are available commercially. Blood collected in ACD for transfusion may be used for certain serotests, but be sure to use the proper blood group and age of cells as specified in the test procedure.

(2) Alsever's Solution, Modified pH 6.1:

Sodium citrate (dihydrate)	0.80 g
Dextrose	2.05 g
Sodium chloride (NaCl) (Reagent Grade)	0.42 g
Distilled water, q.s. to	100 ml

Adjust the pH to 6.1 with 10% (w/v) citric acid. Mix equal parts of Alsever's and blood. Blood collected in Alserver's solution will keep several weeks in the refrigerator.

b. Washing the Cells:

(1) Determine the total volume of RBC suspension required for the number of tests to be performed.

(2) Calculate the minimum volume of packed RBCs required to prepare this total volume of the required concentration of cells by using the following formula:

$$\text{Packed cell volume} = \frac{\text{Total volume required} \times \%\ \text{desired}}{100\%}$$

EXAMPLE: If you need 75 ml of a 2% RBC suspension:

$$\text{Packed cell volume} = \frac{75\ \text{ml} \times 2\%}{100\%}$$
$$= \frac{150\ \text{ml}}{100}$$
$$= 1.5\ \text{ml of packed cells needed.}$$

(3) Put a quantity of blood at least three times the required packed cell volume in a graduated centrifuge tube. The actual volume should take into consideration the amount of anticoagulant in the blood.

(4) Add about 10 volumes of normal saline and mix gently. Centrifuge at 1500–2000 rpm for 5 minutes.

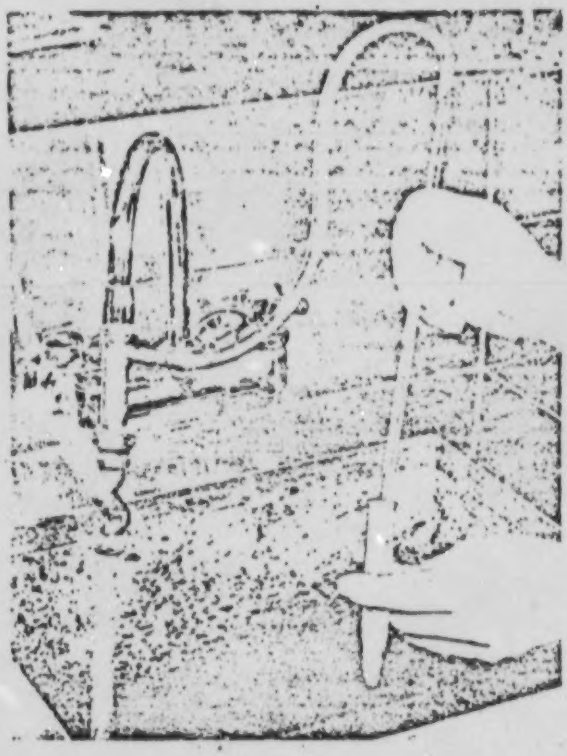


Figure 4–1. Aspiration of Supernate With a Faucet Filter Pump.

(5) Using a faucet filter pump (Richard's pump) or similar device (see Figure 4-1), siphon off the supernatant fluid and the buffy coat (white blood cells).

(6) Resuspend the RBCs in about 10 volumes of saline and repeat steps 4 and 5 at least two more times. The washing is repeated until supernatant is clear (not blood-tinged). Usually about three washings is adequate. Cells that must be washed more than five times to produce a clear supernatant should be discarded and fresh blood collected and washed.

(7) At the last washing, centrifuge the cells for 15 minutes to pack them firmly. Before removing the supernatant, record the volume of packed cells and then carefully remove the supernatant without disturbing the cells. Resuspend the cells to the desired concentration using the diluent specified in the procedure.

NOTE: Although normal saline is used to wash the cells, the final suspension must be made with the diluent specified in the procedure.

c. Resuspending the Washed, Packed Cells:

(1) Calculate the total volume of RBC suspension that the acquired packed cell volume will prepare by using the following formula:

Total volume =

$$\frac{\text{Observed packed cell volume} \times 100\%}{\text{Desired \% cell concentration}}$$

EXAMPLE: If 2.5 ml of packed cells have been prepared and at least 75 ml of a 2% RBC suspension is required, the 2.5 ml of packed cells will make the following total volume:

$$\text{Total volume} = \frac{2.5\ \text{ml} \times 100\%}{2\%}$$

$$= \frac{250\ \text{ml}}{2}$$

$$= 125\ \text{ml}$$

(2) Calculate the amount of diluent to add to the packed cells as follows:

ml diluent to add =

Total volume that packed cells will prepare
− (minus) packed cell volume.

EXAMPLE: In the example started above,

$$\text{ml diluent to add} = 125\ \text{ml} - 2.5\ \text{ml} = 122.5\ \text{ml}.$$

Therefore, the requirements of the hypothetical serotest that needs 75 ml of a 2% RBC suspension will be met with some RBC suspension to spare.

d. Outline for Preparing RBC Suspension:

(1) Collect the blood (see paragraph 4-2a).

(2) Wash the RBCs at least 3 times with normal saline after first performing the following:

(a) Determine the total volume needed.

(b) Calculate the minumum volume of packed cells that will yield the desired concentration and total volume (see paragraph 4-2b).

(3) At the last washing, firmly pack the cells; record the packed cell volume; and carefully removed the supernatant.

(4) Calculate the total volume of RBC suspension that can be prepared from the actual packed cell volume of washed cells (see paragraph 4-2c).

(5) Subtract the packed cell volume from the total volume to be prepared (see paragraph 4-2c).

(6) Resuspend the packed cells in the appropriate diluent to bring the suspension to the desired concentration (see paragraph 4-2c).

4-3. Serial Dilutions:

a. Significance of Serial Dilutions. As described in chapter 2, paragraph 2-7, the only practical method that the serologist has available for measuring serum antibody levels is to determine the titer (reacting capacity) of the serum against a given antigen. However, to determine this antibody titer, the serologist must first make a planned, progressive or serial dilution of the serum in some diluent, such as saline. These serial dilutions can be performed using pipets or with the microtitration equipment described in chapter 1. However, the serial dilutions described here will be for using pipets and test tubes. Microtitration techniques will be described in chapter 14.

(1) To prepare a serial dilution using pipets and test tubes, diluent is added to a series of tubes and then a measured sample of serum is transferred successively from tube to tube so that each tube contains progressively smaller amounts of serum. After the serum has been diluted, the next step is usually to add some of an antigen (such as an RBC suspension) to each tube and record the highest dilution of serum that still gives a specified antigen-antibody reaction. The serum dilution usually is figured to include the added antigen too. The reciprocal of this highest serum dilution is the TITER of that particular serum against that particular antigen. For example, if the highest serum dilution to react to the extent specified in the test procedure is 1/640, the titer would be reported as Positive—1:640 against that antigen.

(2) Although the antibody titer of a serum is not an absolute measure of the antibody content of the serum, the titer does provide a useful mathematical base for certain serotests. Even the titer of a single serum sample could be diagnostic if the titer is higher than usually expected. Or a disease in question might be ruled out if the serum had no titer or a very low one. However, the best use of antibody titers is when a serotest is performed on two serum samples from the same patient. With two samples collected several days or weeks apart, a rise in titer in the second would be most significant. Assuming that the patient had not been immunized recently with the antigen under study, a rise in titer would most likely indicate the presence of active disease or at least recent disease.

(3) Serial dilutions are also very useful for detecting prozone (antibody excess) reactions in serotests. Sometimes undiluted

serum gives only a very weak reaction, but when the test is performed on serially diluted serum, an optimum proportion of antigen to antibody may be achieved at some higher serum dilution with a stronger reaction noted than with undiluted serum.

(4) Not all serial dilutions are limited to serum. Cerebrospinal fluid is used in some of the same tests as serum. An example of making a serial dilution of an antigen is the assay of human chorionic gonadotropin (HCG) in urine. In two conditions, hydatidiform mole and choriocarcinoma, the amount of HCG in the urine is much greater than during pregnancy. So the serologist determines the titer of an antigen (HCG) in these diseases using antibodies (anti-HCG) that have been produced in rabbits.

b. Types of Serial Dilutions. Most serologic tests are designed so that the serial dilution of serum, if required, follows a relatively simple pattern. One of the more common dilution patterns has the serum diluted in half in each succeeding tube of the series. This pattern is called a twofold dilution. Other tests call for fourfold dilutions wherein each succeeding tube contains one-fourth as much serum as the preceding tube. A tenfold dilution is used in still other tests.

(1) Nearly all serologic tests in this manual use twofold serial dilutions. However, the serologist may modify a twofold dilution to obtain just about any dilution sequence he wants. He does this by making a preliminary dilution of the serum before he starts the serial dilution. For example, if he made a preliminary dilution of the serum of one-to-ten (1:10) and then started a twofold dilution of this, the second tube would have a dilution of 1:20; the third 1:40 et cetera. By varying the starting point of a simple twofold serial dilution a nearly unlimited variety of dilutions can be achieved.

(2) Unfortunately, not all serologic tests use simple serial dilutions. The antistreptolysin-O (ASO) titer (see chapter 9) is a good example of a very complex serial dilution technique. In one modification of the ASO titer, three different preliminary dilutions are made. In a method for determining the titer of human chorionic gonadotropin (HCG) (see chapter 13), the serial dilution of urine starts out as tenfold and then switches in midseries to a fivefold dilution. Consequently, the serologist must be sure to follow all serial dilution procedures carefully but especially these complex ones.

c. Performing a Typical Serial Dilution. In this manual, most procedures that require serial dilutions will be presented in written form with the major steps also outlined diagrammatically in a table. The examples given in this chapter will be similar to those used throughout the manual.

(1) Preliminary Steps. Before starting the serial dilution, examine the test procedure and assemble the equipment and reagents required. Special attention should be paid to the following:

(a) Water baths or heating blocks; operating at the proper temperature.

(b) Test tubes; clean and of specified size.

(c) Pipets; generally the smallest usable size.

(d) Diluent; saline or modification, as specified.

(e) Antigen suspension; prepared as specified and at the specified temperature before use.

(f) Serum or other specimens; fresh or inactivated as specified. For frozen serum, be sure it is thawed, mixed gently and allowed to warm to room temperature before inactivation. Also be sure all specimens are at room temperature before pipetting.

(2) Procedure, Example:

(a) Place seven test tubes (12 × 75 mm) in a rack. Label the first tube with the patient's name, et cetera, and number each tube consecutively. Some test tube racks have numbered slots so if the test tubes do not have to be removed from the rack during the test, only the first tube needs to be labeled. However, a good habit is to label all tubes, if practical.

(b) With a 5 ml serological pipet used with the point-to-point technique or with an accurately calibrated, syringe-type automatic pipetter, put 0.5 ml of normal saline in each tube.

(c) In a separate test tube, make a preliminary 1:5 dilution of fresh serum (not inactivated) by adding 0.2 ml of serum to 0.8 ml of normal saline. Use a separate pipet for each reagent and the smallest, usable size.

(d) With a 1.0 ml serological pipet, mix the contents of the preliminary 1:5 dilution of serum by carefully sucking the mixture into the pipet and then gently blowing it back into the test tube. Repeat the mixing at least 3 times, but do not create bubbles in the mixture. After mixing, suck slightly more than 0.5 ml of mixture into the pipet and lower the meniscus exactly to the 0.5 ml mark. Remove droplets on the tip of the pipet by rubbing the pipet around the inside of the test tube as the pipet is withdrawn. The pipet is not wiped dry as it would be in normal pipeting technique.

(e) Transfer the 0.5 ml of 1:5 dilution to the first tube and blow out the last drop. NOTE: For greatest accuracy, this pipet should now be discarded and a new pipet used to mix and dilute each tube. However, for practical purposes the same pipet is used throughout the serial dilution. The important considerations are to be consistent and at the same time try to remove as much external fluid as possible from the pipet as it is removed from each tube.

(f) Using the same pipet, mix and transfer 0.5 ml from tube #1 to tube #2 and repeat through tube #6. After mixing, discard 0.5 ml from tube #6. Tube #7 is the Antigen Control and does not receive any serum.

(g) Examine the fluid level of each tube. Each should have 0.5 ml if the serial dilution has been performed properly.

(h) Add 0.5 ml of antigen, such as a bacterial or RBC suspension to each tube including #7.

(i) Incubate the tubes and read the reactions as specified for the particular procedure. The preceding procedure is presented diagrammatically in table 4–1.

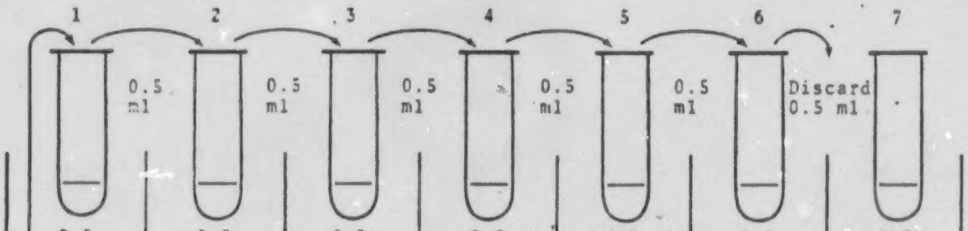
d. Calculating the Titer. As defined, the antibody titer is the reciprocal of the highest serum dilution that still gives a positive antigen-antibody reaction. Unfortunately, this definition must be qualified because some tests have been designed that included the addition of the antigen before figuring the serum dilution while other tests use the dilutions before the antigen is added. Therefore, be sure to follow the system used for each given serotest.

(1) The serum dilution of a given tube is most easily determined by considering the ratio of serum to the total volume in the tube as a fraction. This most easily accomplished if the numerator is converted to one. For example, in the preliminary dilution made in the sample serial dilution given in paragraph 4–3c(2), 0.2 ml of serum is mixed with 0.8 ml of saline for a total volume of 1.0 ml. The dilution of serum is 0.2 ml serum/1.0 ml total volume which is proportional to 1 ml serum/5 ml total volume or a 1:5 dilution.

(2) Once the dilution of the preliminary tube is known, calculating the dilution of

Table 4–1. Example, Typical Serial Dilution Procedure.

Table 4-1. Example, Typical Serial Dilution Procedure.



Tube Number	1	2	3	4	5	6	7
Saline, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5 milliliters of:						
Serum, ml	1:5 (From separate tube	1:10	1:20	1:40	1:80	1:160	None (Control)
Serum Dilution After Transfer	1:10	1:20	1:40	1:80	1:160	1:320	---
Appropriate Antigen Suspension, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total Volume, ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final Dilution of Serum	1:20	1:40	1:80	1:160	1:320	1:640	---

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other tubes in the series is simply a matter of multiplying fractions and can usually be performed mentally. For example, from the example in paragraph 4-3c, a twofold dilution is made starting with the preliminary 1:5 dilution. Therefore, before the antigen is added, the serum dilution in the first series would be:

$$\frac{1}{5} \times \frac{1}{2} = \frac{1}{10} \text{ or } 1{:}10$$

After an equal volume of antigen is added, the final dilution of serum in the first tube becomes:

$$\frac{1}{10} \times \frac{1}{2} = \frac{1}{20} \text{ or } 1{:}20$$

Each succeeding dilution can be calculated in this fashion but be sure to check the test procedure for whether the antigen is considered as part of the serum dilution or not. Another important step to remember is to always represent the serum to total volume ratio as one part of serum to whatever total volume exists; then the reciprocal of this fraction will be expressed in the proper term for reporting titers as one part of serum in some total volume, such as 1:640, 1:1280, et cetera.

4-4. Reporting Results of Serologic Tests. As soon as practical after the completion of a serotest, the results should be recorded on the patient's laboratory slip and dispatched to the appropriate office. The proper way to report the results is provided with each serotest in this manual. However, there are certain general principles that should be described.

a. Tests Without Serial Dilution. Several serotests do not require a serial dilution as part of the test procedure. These tests are usually reported simply as either POSITIVE or NEGATIVE. However, a variation of the positive-negative report is used with tests for syphilis. Syphilis tests are reported as either REACTIVE or NONREACTIVE.

b. Tests with Serial Dilutions:

(1) Positive (Reactive) Tests. Positive results from tests involving serial dilutions are usually reported as positive (or reactive) followed by the titer. For example, a test result might be reported as follows: Positive—1;20, Pos-titer 1:20; or some similar format. CAUTION: Before reporting any titer, be sure that the test has been read as specified in the test procedure. This precaution must be emphasized because many serotests specify some degree (strength) of antigen-antibody reaction on which to base the titer. Usually the reactions are rated from 1 to 4+ with 1+ being a very weak reaction and 4+ the strongest possible. Using this system, a given test procedure may specify that the titer is the highest dilution of serum to give a 2+ or greater reaction. A 1+ reaction might be reported as BORDERLINE so be sure to follow the procedure in determining the end point of a reaction.

(2) Negative (Nonreactive) Tests. Do NOT report a serotest as NEGATIVE if none of the tubes in a serial dilution show a reaction. The best way to indicate these results is to name the antigen and indicate that the reaction may have been at some point below the lowest serum dilution used in the test. For example, a report might read: *Salmonella typhi* H—less than 1:20. This report would indicate to the physician that a 1:20 dilution was the lowest serum dilution tested against that particular antigen but does not rule out the possibility that a 1:5, 1:15, or even 1:19 dilution might have reacted. The fact is that these lower dilutions have not been tested so it would be unfair to suggest they had been tested by calling the results negative. Each test has been designed so that titers less than the first tube of the series are usually insignificant but unless they are tested they should not be reported.

Chapter 6

TESTS FOR SYPHILIS

SECTION A—THE DISEASE

6-1. Introduction:

a. Venereal Route of Infection. Syphilis is a contagious venereal disease caused by the spirochete, *Treponema pallidum.* The usual site of invasion of the treponeme is the male's glans penis and female's cervix, but other parts of the genitalia may be the invasion site. Although the disease is contracted primarily during direct sexual contact, invasion may be through other mucous membranes, such as of the mouth or anal region. Even abrasions or cuts in the skin of other parts of the body may serve as sites of entry of the treponeme—a fact that makes syphilis an occupational hazard for members of the medical profession.

b. Congenital Syphilis. Another infection route—one that justifiably generates a considerable amount of laboratory work—is the transmission of treponemes from a syphilitic pregnant woman across the placental barrier to her developing fetus. The resulting disease is referred to as congenital syphilis. This condition may (and should) be treated in utero by treating the mother as soon as her infection is detected. Many of these cases result in abortions and stillbirths. However, some infants are born alive with signs of syphilis already present. Some syphilitic infants are born without overt signs of the disease, but disfigurement, blindness, deafness, and other complications may develop unless treatment is started as soon as possible.

c. Treatment. Fortunately, the treatment of all types of syphilis, including congenital syphilis, is adequately performed with penicillin. Only rarely have cases failed to be completely cured by one or two doses of the drug. Therefore, the persistent (and increasing) incidence of syphilis is not due to inadequate therapy, but the disease persists due to changing sociological standards and—of prime interest to the serologist—frequent difficulties in diagnosing the disease. These diagnostic difficulties are compounded by the fact that untreated venereal syphilis may go through four distinct stages, but exact stages and their manifestations are not predictable from patient to patient.

6-2. Stages of Untreated Venereal Syphilis:

a. Primary Syphilis. After an incubation period of about 3 weeks (range: about a week up to 3 months), a primary chancre appears at the site of invasion of the treponemes. Usually only one chancre is found. The only other findings may be enlarged lymph glands in the region of the chancre. This highly infectious chancre disappears in about a month even in untreated cases—more quickly, if treated—but the treponemes are still in the bodies of untreated cases.

(1) Diagnosis of primary syphilis is accomplished mainly by darkfield microscopic examination of material from the chancre for treponemes. Darkfield microscopy and the morphology of the treponemes will not be described in this manual. Although a small percentage of cases have detectable levels of antibodies during the primary stage of the disease, a nonreactive serologic test for syphilis at this time does not rule out the disease.

(2) Usually, after the apparent spontaneous resolution of the primary chancre in untreated syphilis, there is a period when no visible signs of the disease are present. The serologic tests are nearly always reactive during this period, which may last up to several months. Occasionally, however, the secondary stage of syphilis may start before the primary lesion has disappeared.

b. Secondary Syphilis. This stage of syphilis usually starts before or within a few months after the disappearance of the primary lesion. It is characterized by fever, malaise, mucous patches in the mouth, condylomata lata of the ano-genital region, and a localized or generalized rash. All of these lesions are highly infectious. In some patients, these lesions last only a few days, but they may last up to 1 year. Periodic recurrences of these signs and symptoms may occur for a couple of years.

(1) Diagnosis of secondary syphilis may be made by darkfield examination of the lesions. In addition, nearly 100 percent of

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Material in Chapter 6 was adapted from *Manual of Tests for Syphilis*, 1969, PHS Publication No. 411.

patients in this stage will have reactive serologic tests on serum.

(2) With treatment, the symptoms of secondary syphilis disappear rapidly. Even without treatment, symptoms eventually disappear, and the disease usually enters a latent period unless the disease progresses directly into the tertiary stage of syphilis.

c. Latent Syphilis. This stage of the disease is characterized by reactive serologic test for syphilis on serum, but the cerebrospinal fluid (CSF) must be nonreactive. If the CSF is reactive, the disease has already entered the tertiary stage. No other manifestations of syphilis are present during the latent stage, which may last several years or for the life of the patient. An additional feature of latent syphilis is that the disease is no longer communicable to another individual, EXCEPT transplacentally to a fetus. A given patient with latent syphilis may have his disease develop in one of three ways.

(1) In about one-fourth of the untreated patients, the latent period (that is, a reactive serologic test) may last for life without any other manifestations of syphilis.

(2) Another fourth of the patients experience what might be considered a spontaneous cure. In other words, their serologic tests become nonreactive and no additional manifestations of syphilis ever develop. Unfortunately, some of these apparently spontaneous cures may have developed into the most serious stage of syphilis—tertiary (or late) syphilis—even though the sera of these patients may be nonreactive in serologic tests. Usually these cases can be detected because they develop reactive serologic tests on their spinal fluid.

(3) In addition to the apparently spontaneous cures that really have developed tertiary syphilis, about half of all cases of latent syphilis progress to tertiary syphilis unless they are treated at some earlier stage.

d. Tertiary (Late) Syphilis. The manifestations of tertiary syphilis are varied and generally do not appear for several years after the primary infection. Cases may resemble an apparent latent stage for up to 40 years before symptoms appear. Tertiary syphilis may involve the central nervous system with the possibility of insanity, blindness, and other neurological problems. The cardiovascular system is also frequently affected with the most serious complication being the development of an aortic aneurysm which may rupture and result in nearly instantaneous death. In addition, lesions may be produced in the skin, bone, and internal organs. These lesions are not infectious, EXCEPT that a developing fetus may become infected even during this late stage of the disease. All of these serious complications of syphilis make the early diagnosis and treatment of syphilis a professional challenge. Fortunately, most cases of tertiary syphilis, as well as secondary and latent syphilis, will have reactive serologic tests for syphilis. In addition, the spinal fluid of cases with central nervous system involvement will usually have reactive tests. These facts dictate a major role for the serologist in the diagnosis of syphilis. In fact, serologic tests for syphilis are the most frequently performed serologic tests.

6-3. Types of Serologic Tests for Syphilis. The serodiagnosis of syphilis is based on the detection of two broad categories of antibodies. Most of the tests (especially the screening tests) are based on the detection of antibodies against antigens that have no logical relationship with the causative treponemes. These tests are referred to as nontreponemal or reagin tests. In addition, several tests have been developed that use antigens that are treponemal in origin. Examples of both types will be presented in this chapter.

a. Nontreponemal (Reagin) Tests. The antigen used in most of these tests is an extract of beef heart, known as cardiolipin. The cardiolipin may be mixed with lecithin and cholesterol for use in microscopic flocculation tests such as the VDRL and USR tests. Cardiolipin has also been combined with carbon particles (charcoal) to produce macroscopic tests, such as the RPD tests. In addition, various nontreponemal antigens have been used in complement fixation as well as several other varieties of flocculation tests. These nontreponemal tests are usually referred to collectively as a "blood test," "serology," or, more specifically "STS," which translates to "serologic test for syphilis." These tests are also frequently called a "VDRL," but "VDRL" actually refers to one particular reagin test.

(1) The syphilitic antibodies against nontreponemal antigens are considered to be antibody-like, because the antigens in use cannot stimulate their production, so they are not true antibodies. They are also called reagins or reaginic antibodies to differentiate them from treponemal antibodies.

(2) Because nontreponemal (reaginic) antibody production is not specifically stimulated by the treponemes, several other conditions can cause these antibodies to be produced. Malaria, leprosy, hepatitis, and cer-

tain immunizations are just a few of the conditions that can cause reactive nontreponemal tests at varying rates. These results are called biological false positives—BFP, for short. These BFPs, along with the social implications of syphilis, are the reason that serologic tests for syphilis are reported as "Reactive, Weakly Reactive or Nonreactive" and NOT "Positive" or "Negative." These conditions causing BFPs must be differentiated from actual treponemal infections. Reactive tests due to related treponemal infections, such as bejel, pinta, and yaws, should not be classified as BFPs. Fortunately, tests on spinal fluid rarely produce BFPs; a reactive usually indicates syphilis—in fact, tertiary syphilis.

(3) In addition to their usefulness as screening tests for syphilis, nontreponemal (reagin) tests are helpful in following the effectiveness of therapy. The antibody titer in these tests drops rapidly following successful treatment of primary syphilis. In secondary syphilis, the titer drops more slowly but usually will eventually become nonreactive.Unfortunately, titers in treated latent and tertiary syphilis may remain fairly constant for years or life. By contrast, titers of the tests using treponemal antigens do not normally drop; therefore, these reagin tests are at least useful in following treatment of the early stages of syphilis.

b. Treponemal Tests. Serologic tests for syphilis that utilize the treponeme as the source of antigen generally require more sophisticated equipment and techniques than reagin tests. However, the main advantage of treponemal tests, such as the Fluorescent Treponemal Antibody-Absorption Test (FTA-ABS), is that biological false positives are essentially eliminated. In fact, the FTA-ABS test is frequently used to confirm or reject the findings of one of the nontreponemal tests when these results are questionable. Although the FTA-ABS test is currently the most widely used treponemal-type test, two other tests are still in use, but declining. These are the Treponema Pallidum Immobilization Test (TPI) and the Reiter-Protein Complement Fixation Test (RPCF). Only the FTA-ABS will be presented in detail in this chapter.

SECTION B—TESTS ON SERUM

6–4. Rapid Plasma Reagin (RPR) "18 MM Circle" Card Test:

a. Principle. Unheated serum (0.05 ml) is spread within an 18 mm circle on a plastic-coated card. A drop (1/60 ml) of carbon-containing RPR antigen is added to the serum without mixing. The card is rotated on a slide rotator circumscribing a $^{3}/_{4}$-inch circle for 8 minutes at 100 rpm. Macroscopic flocculation of the carbon particles indicates the presence of reaginic antibodies in the serum.

b. Reagents and Equipment:

(1) Patient's serum, unheated.

(2) Control sera of known reactivity, available commercially or collected locally.

(3) RPR card test kit, 18 mm circle, available through federal supply channels complete with cards, capillaries for dispensing serum, and antigen suspension with a dispensing needle. NOTE: Several other types of RPR kits are available from Hynson, Westcott, and Dunning, Inc. These kits, including one for hand rotation, may more adequately fulfill the objectives of a given laboratory. Be sure to follow all instructions for the test selected.

(4) Slide rotator, circumscribing a $^{3}/_{4}$-inch circle and set at 100 rpm.

(5) Humidifying cover with moist blotter to cover cards during rotation.

c. Preliminary Steps:

(1) Preparation of Sera and Antigen:

(a) Collect patient's serum. The serum may be left on the clot after sedimentation.

(b) Allow all sera, including controls, and the RPR antigen to warm to room temperature for about 30 minutes prior to testing.

(2) Testing of Delivery Needles. When the antigen is at room temperature, test the accuracy of the dispensing needle (20-gage without bevel). This needle must deliver 60 ±2 drops of antigen suspension/milliliter. Crimp or ream the bore of the needle, as appropriate, and retest until an accurate drop is dispensed.

(3) Pretesting of RPR Antigen. Check the suitability of the antigen with unheated control sera of known reactivity by using the procedure in paragraph d below. If the expected results are obtained, proceed with testing the patients' sera. Discard antigen lots that fail to react properly.

d. Procedure:

(1) Place 0.05 ml of unheated serum on a circle of the test card using a calibrated capillary, serological pipet or Dispenstir (Hynson, Westcott and Dunning, Inc.).

(2) Spread the serum over the entire surface of the 18 mm circle with the wooden sticks or Dispenstirs. Avoid scratching the card.

(3) Gently shake the antigen suspension and dispense one drop (1/60 ml) of antigen to

each serum with the needle held vertically. Do not stir the antigen-serum mixture.

(4) Rotate the card for 8 minutes at 100 rpm. Be sure to use a humidifying cover to prevent drying of the mixture.

(5) Immediately following rotation, examine the cards under a light for macroscopic flocculation of the carbon particles. This reading must be made while the mixture is wet. Brief tilting of the card may aid interpretation of questionable readings.

e. Reporting Results. If the control sera produce the expected results, report the patients' sera as follows:

(1) Sera producing no flocculation of the carbon particles or only a slight roughness—Nonreactive.

(2) Sera producing flocculation (clumping) of the carbon particles—Reactive, preferably along with a titer. Procedures for a quantitative card test are included with the manufacturer's instructions.

f. Sources of Error:

(1) Failure to follow manufacturer's instructions, especially storage instructions and expiration dates of the antigen, may produce invalid results.

(2) Scratched cards may resemble flocculation.

(3) All reagents must be at room temperature for testing. Cold reagents may decrease test sensitivity; if they are too warm, the test may be hypersensitive.

(4) Dispensing needles must be accurate. Each serologic test may use a different size of antigen drop so don't interchange needles without testing them. Before storing the needle, be sure to blow all fluid from it, because dried antigen may alter the drop size, but do not wipe the needle as this removes the silicone coating.

(5) Do not confuse the 18 mm circle test with the 14 mm card test which uses different volumes of antigen and plasma. Still other tests for syphilis use 14 mm ceramic or paraffin rings on glass slides.

g. Discussion:

(1) Biological false positives are possible with this test.

(2) Plasma collected with a variety of anticoagulants may be substituted for serum. Check the manufacturer's instructions for acceptable anticoagulants.

6-5. Qualitative VDRL (Cardiolipin Microflocculation) Slide Test:

a. Principle. Cardiolipin antigen (1/60 ml) containing lecithin and cholesterol is added to 0.05 ml of inactivated serum on a slide with 14 mm paraffin or ceramic rings. The slide is rotated for 4 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100X magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the serum.

b. Reagents and Equipment:

(1) Patient's serum, inactivated.

(2) Control sera of known reactivity, inactivated, available commercially or collected locally.

(3) VDRL (Cardiolipin Microflocculation) slide test antigen.

(4) VDRL buffered saline, pH 6.0 ±0.1.

(5) Pipets, capillaries, or Dispenstirs.

(6) Slides, with 14 mm paraffin or ceramic rings. NOTE: Slides, such as boerner slides, are not recommended for this test.

(7) Bottle, 30 ml, round, glass-stoppered, with flat innerbottom. NOTE: Be sure the innerbottom is flat.

(8) Water bath, 56°C.

(9) Slide rotator, set at 180 rpm.

(10 Ringmaker, if paraffin-ringed slides are to be used.

(11) Syringe, 1 ml.

(12) Needles, hypodermic, 18-gage without bevel or 23-gage with bevel, pretested to deliver 1/60 ml/drop.

c. Preliminary Steps:

(1) Preparation of Serum:

(a) Inactivate clear sera at 56°C for 30 minutes. Sera, such as control sera, that have been inactivated previously or that were inactivated more than 4 hours before being tested must be reheated for 10 minutes at 56°C.

(b) Cool all sera to room temperature before testing.

(2) Preparation of VDRL Antigen Emulsion:

(a) Pipet 0.4 ml of buffered saline to the bottom of the 30-ml bottle.

(b) Load the bottom half of a 1-ml serological pipet with 0.5 ml of VDRL antigen and introduce the tip of the pipet into the upper third of the 30-ml bottle.

(c) Start rotating the bottle at about three times/sec on a flat surface so that the center of the bottle circumscribes a circle about 2 inches in diameter. Do not make the buffer splash.

(d) Add the antigen dropwise while rotating the bottle, blowing out the last drop. This addition should take about 6 seconds.

(e) Continue rotating the bottle for 10 seconds more.

(f) Add 4.1 ml of VDRL buffered saline to the emulsion in the bottle.

(g) Place the glass stopper in the bottle and shake the bottle vigorously for 10 seconds. The fluid should be thrown from the bottom to the top of the bottle.

(h) Use the antigen only on the day of preparation.

(i) This quantity of antigen is adequate for about 250 tests. If more is needed, multiply the quantities of antigen and buffer and prepare a larger batch. Do NOT make smaller batches than the quantities indicated above.

(3) Testing of Delivery Needles:

(a) Gently mix the antigen emulsion and fill a 1-ml syringe fitted with a 23-gage needle with bevel or 18-gage without bevel (bevel removed).

(b) Holding the bevel of the 23-gage needle HORIZONTALLY (or the 18-gage needle VERTICALLY), determine the number of drops of antigen emulsion/milliliter. The needle must deliver 60 drops ± 2 drops/milliliter.

(c) Adjust the bore of the needle until the 60 drops/ml are delivered.

(4) Pretesting of VDRL Antigen Emulsion:

(a) Test known control sera (Reactive, Weakly Reactive, and Nonreactive) using the procedure in paragraph d below. Be sure the sera have been properly inactivated before testing.

(b) Discard antigen emulsions that do not produce the expected results.

d. **Procedure:**

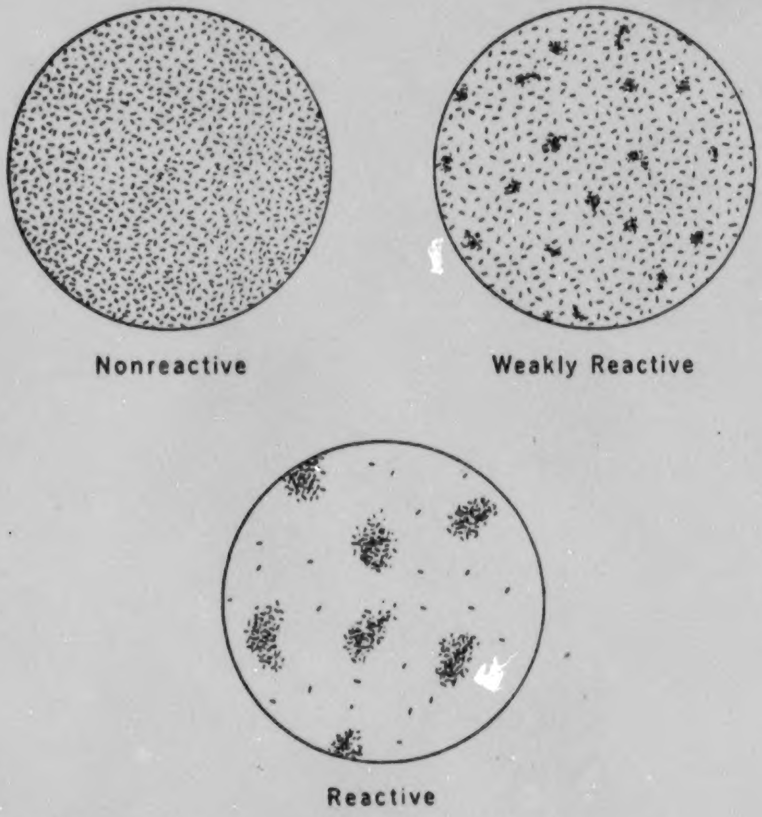
(1) Pipet 0.05 ml of inactivated serum to a 14 mm paraffin or ceramic ring on a slide. A calibrated capillary pipet or Dispenstir may also be used.

(2) Add one drop (1/60 ml) of antigen to each serum with a pretested 18- or 23-gage needle.

(3) Rotate the slide for 4 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(4) Read the tests at 100× magnification (10× ocular and 10× objective) for flocculation of the antigen particles. See figure 6-1 for artist's conceptions of the reactions.

e. **Reporting Results.** Before any results can be reported, the control sera must produce the expected results. If the controls indicate valid results, report the patient's sera according to table 6-1, preferably along with the



Nonreactive

Weakly Reactive

Reactive

Figure 6-1. VDRL Slide Test Reactions.

Table 6-1. Reporting Results of VDRL Slide Tests.

Table 6-1. Reporting Results of VDRL Slide Tests.

Reading	Report
No clumping or very slight roughness.	Nonreactive (N).
Small clumps.	Weakly Reactive (WR).
Medium and Large Clumps.	Reactive (R).

titer of all Reactive, Weakly Reactive, or "rough" sera. See paragraph 6-6 for the VDRL quantitative test.

f. Sources of Errors:

(1) All reagents must be at room temperature for testing. Cold reagents may decrease test sensitivity; if they are too warm, the test may be hypersensitive.

(2) This test must be performed only on clear serum. Cloudy serum or heated plasmas may contain confusing precipitates.

(3) Biological false positives are possible.

(4) Prozonal reactions may occur; therefore, all Weakly Reactive, as well as "rough" reactions, should be titered to detect potentially high titered sera. See paragraph 6-6 for methods.

(5) Mix the antigen emulsions frequently to insure an even suspension of antigen particles.

(6) The pH of the buffered saline is critical. Alterations may affect test sensitivity.

(7) The antigen emulsion should only be used on the day it is prepared. For a test using a more stable antigen see the USR test in paragraph 6-7.

6-6. Quantitative VDRL (Cardiolipin Microflocculation) Slide Test:

a. Principle. Reactive, Weakly Reactive, and "rough" sera are serially diluted in 0.9% saline. Each serial dilution is tested as if it were an individual serum by the VDRL slide test. The highest serum dilution to produce a Reactive (not Weakly Reactive) Result is reported as the titer.

b. Reagents and Equipment. All items listed for the qualitative VDRL slide test in paragraph 6-5b are needed, plus the following:

(1) Saline, 0.9%.

(2) Test tubes, 12 × 75 mm.

c. Preliminary Steps:

(1) Prepare sera and the antigen emulsion, test the delivery needle, and pretest the antigen emulsion according to instructions in paragraph 6-5c.

(2) Prepare a twofold serial dilution of all Reactive, Weakly Reactive, and "rough" patients' sera as follows:

(a) Place five test tubes (12 × 75 mm) in a rack.

(b) Put 0.5 ml of 0.9 percent saline in each tube.

(c) Pipet 0.5 ml of inactivated serum to the first tube. NOTE: For greatest accuracy, this pipet may be discarded and a new one used to mix and transfer the saline-serum mixture to each succeeding tube.

(d) Mix by aspiration and transfer 0.5 ml of saline-serum mixture to the second tube. Repeat this through the fifth tube. Leave the pipet in the fifth tube in case more dilutions are needed. The resulting dilution is twofold starting with 1:2 in the first tube. This dilution scheme is shown graphically in table 6-2.

d. Procedure. Test each dilution like an individual serum by the VDRL slide test described in paragraph 6-5d. Continue the serial dilution if all five dilutions should be reactive.

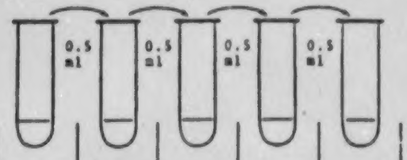
e. Reporting Results. The highest dilution of serum producing a Reactive (not Weakly Reactive) result is reported as the titer. Examples of quantitative VDRL test results are shown in table 6-3.

6-7. Unheated Serum Reagin (USR) Test:

a. Principle. Unheated serum (0.05 ml) is placed on a 14 mm paraffin- or ceramic-ringed slide. A drop (1/45 ml) of a cardiolipin antigen containing choline chloride to inacti-

Table 6-2. Quantitative VDRL Serum Dilutions.

Table 6-2. Quantitative VDRL Serum Dilutions.



Tube Number	1	2	3	4	5
Normal Saline, ml	0.5	0.5	0.5	0.5	0.5
Serum, ml		0.5 milliliters of:			
	0.5	1:2	1:4	1:8	1:16
Final Serum Dilution	1:2	1:4	1:8	1:16	1:32

Table 6–3. Example, Reports of Quantitative VDRL Serum Dilutions.

Table 6-3. Example, Reports of Quantitative VDRL Serum Dilutions.

Specimen	Reaction with serum diluted 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Report
Serum A	R	R	R	R	R	R	WR	N	Reactive, titer 1:64
Serum B	R	R	R	WR	N	N	N	N	Reactive, titer 1:8
Serum C	WR	N	N	N	N	N	N	N	Reactive, titer undiluted only.
Serum D	WR	R	R	R	R	R	R	N	Reactive, titer 1:128

vate serum complement and EDTA to preserve the antigen emulsion is added to each serum without mixing. The slide is rotated on a slide rotator circumscribing a 3/4-inch circle for 4 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100× magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the serum.

b. Reagents and Equipment. All items listed for the qualitative VDRL slide test in paragraph 6–5b are needed, except that the needle must deliver 1/45 ml/drop and the water bath is not needed.

(1) Centrifuge, angle-head with tachometer, capable of achieving an rcf of 2000 g.

(2) Tubes, stainless steel, for centrifuge.

(3) Phosphate (0.02 M) Merthiolate (0.2%) solution. Dissolve 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4, and 1.00 g Merthiolate in distilled water in a 500-ml volumetric flask. When dissolved, q.s. to 500 ml with water, the pH should be 6.9. Store in the dark at room temperature. Stable up to 3 months.

(4) Choline chloride solution, 40%. Dissolve the entire contents of a 250 g bottle of choline chloride in 625 ml of distilled water. Filter and store at room temperature. Stable 1 year. Refilter if needed. NOTE: Use the entire bottle due to the hygroscopic nature of choline chloride.

(5) EDTA, 0.1 M. Q.s. 3.72 g EDTA (disodium salt) to 100 ml with distilled water. Stable 1 year.

(6) USR antigen emulsion (see paragraph c(3) below).

c. Preliminary Steps:

(1) Preparation of Serum. Clear, unheated serum at room temperature is needed.

(2) Preparation of USR Antigen Resuspension Solution. Prepare this solution each time antigen emulsion is to be prepared. Be sure the reagents are not outdated. Prepare a volume equal to the volume of antigen to be prepared. To prepare 10 ml of resuspending solution, combine the following:

EDTA (0.1 M)	1.25 ml
Choline chloride (40%)	2.5 ml
Phosphate (0.02 M), Merthiolate (0.2%)	5.0 ml
Distilled water	1.25 ml

(3) Preparation of USR Antigen Emulsion:

(a) Prepare a large batch of VDRL slide test antigen according to instructions in paragraph 6–5c(2). For example, 100 ml of antigen emulsion may be prepared by dropping 10 ml of antigen into 8 ml of buffered saline in a 250 ml glass-stoppered bottle. After an additional 10 sec of rotation, add 82 ml of buffered saline, stopper the bottle and shake according to instructions for the VDRL antigen.

(b) Centri[illegible]ge measured amounts of the VDRL antigen emulsion at an rcf of 2000 g for 15 minutes (time only when the desired speed is reached). See paragraph 1–7 for calculation of the desired rpm.

(c) Decant the supernate OPPOSITE the side containing the sediment. Wipe excess fluid from the inside of the tube with gauze without disturbing the sediment.

(d) Using freshly prepared resuspension solution, resuspend the sediment in a volume equal to that of the VDRL antigen originally centrifuged.

(e) Pool all tubes of antigen in a tightly stoppered bottle and gently shake the bottle to produce an even suspension. NOTE: The antigen is now ready for pretesting with known control and for future use. The antigen is usually stable for at least 6 months if kept at 3°C-10°C. Remove only an aliquot for each test run, and include control sera with each run. The antigen aliquot must be at room temperature for testing.

(4) Testing of Delivery Needle:

(a) Gently mix the antigen emulsion and fill a 1-ml syringe fitted with an 18-gage needle with the bevel removed.

(b) Holding the needle vertically, determine the number of drops/milliliter. This test requires 45 drops ± 1 drop/milliliter.

(c) Adjust the bore of the needle until 45 drops/ml are delivered.

(5) Pretesting of USR Antigen Emulsion:

(a) Test known control sera (Reactive, Weakly Reactive, and Nonreactive) using the procedure in paragraph d below. All sera and the antigen must be at room temperature for testing.

(d) Discard antigen emulsions that do not produce the expected results.

d. Procedure:

(1) Pipet 0.05 ml of clear, unheated serum at room temperature to a 14 mm paraffin or ceramic ring on a slide. A calibrated capillary or Dispenstir may be used.

(2) Add one drop (1/45 ml) of USR antigen emulsion at room temperature to each serum with a pretested 18-gage needle.

(3) Rotate the slide for 4 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(4) Read the tests at 100× magnification (10× ocular and 10× objectives) for flocculation of the antigen particles. See figure 6-1 for artist's conceptions of the reactions.

e. Reporting Results. Results are reported in the same manner as for the qualitative VDRL slide test given in paragraph 6-5e and table 6-1.

f. Sources of Error. Sources of error are the same as for the qualitative VDRL slide test with the following additions:

(1) Keep the USR antigen emulsion in the refrigerator at all times. Remove only as much as is needed for a given batch of tests. Allow this aliquot (as well as refrigerated sera) to warm to room temperature for about 30 minutes before testing.

(2) Check the antigen during each test run with known controls. Discard antigen lots when they fail to produce the expected results.

(3) The serum is not heated for the USR test. The choline chloride in the antigen emulsion inactivates the complement.

6-8. Fluorescent Treponemal Antibody-Absorption (FTA-ABS) TEST:

a. Principle: First, the patient's serum is absorbed with an extract of Reiter treponemes to adsorb antibodies that are common to both saprophytic and pathogenic treponemes. The adsorbed serum is then added to the Nichols strain of *Treponema pallidum* that has been dried on the slide and fixed with acetone. After incubation at 37°C, excess serum is rinsed from the slide with phosphate buffered saline (PBS). Then, fluorescein-labeled anti-human globulin (conjugate) is added to the *T. pallidum* smear, and the slides are incubated again at 37°C. At the end of this incubation period, excess conjugate is rinsed from the slide with PBS. The slides are coverslipped with a glycerine mounting medium and examined by fluorescent microscopy using the appropriate exciter and barrier filters. The presence of treponemal antibodies is indicated by a greenish fluorescence of the treponemes. The observations are compared with the fluorescence due to known control sera.

b. Reagents and Equipment:

(1) The following items are available from several commercial sources and are usually purchased rather than prepared in the laboratory. Store and reconstitute these according to the manufacturer's instructions.

(a) *Treponema pallidum* (Nichols Strain) (FTA-ABS) antigen, lyophilized.

(b) Fluorescein-labeled antihuman globulin (conjugate).

(c) Sorbent, FTA-ABS.

(d) Reactive (4+) control serum.

(e) Minimally reactive (1+) control serum (prepared from 4+).

(f) FTA nonspecific serum control.

(2) Patient's serum, inactivated.

(3) Phosphate buffered saline (PBS), pH 7.2 ± 0.1.

NaCl	7.650 g
Na_2HPO_4	0.724 g
KH_2PO_4	0.210 g
Distilled water	q.s. to 1 liter

Check the pH of the PBS frequently and discard lots that vary by more than ± 0.1 from pH 7.2. NOTE: The mixed chemicals for PBS of the proper pH are also available commercially in powder form.

(4) Tween 80 (Atlas Chemical Industries, Inc.).

(5) PBS containing 2 percent Tween 80. Heat PBS and Tween 80°C to 56°C. Pipet 2 ml of Tween 80 to 98 ml of PBS with rinsing. Store in the refrigerator. Discard if a precipitate forms or the pH changes.

(6) Mounting medium, 1 part PBS plus 9 parts glycerine.

(7) Acetone, reagent grade, dry.

(8) Microscope slides, frosted end, about 1 mm thick.

(9) Cover slips, No. 1, 22 × 40 mm.

(10) Staining dish, with removable slide carrier.

(11) Pipets, serological, 0.2 ml.

(12) Pipets, capillary.

(13) Test tubes, 12 × 75 mm.

(14) Incubator, bacteriological, at 35°C-37°C.

(15) Fluorescent microscope with darkfield condenser and UV light source.

(16) Moist chamber. A plastic, 25-capacity, slide box with a tight cover may be used. Set the box on end with wet gauze at the bottom end to keep the reactants on the slide uppermost.

(17) Water bath, 56°C.

(18) Diamond-point pencil.

(19) Bibulous paper.

(20) Loop, bacteriological, 2-mm from 26-gage platinum.

(21) Immersion oil, low-fluorescence.

c. Preliminary Steps:

(1) Preparation of Sera. Control sera should be reconstituted and stored according to the manufactuer's instructions. All sera, including control sera, must be heated at 56°C for 30 minutes before testing. Sera that have been heated previously should be reheated for 10 minutes at 56°C. Do NOT heat the conjugate.

(2) Preparation of Minimally Reactive (1+) Control Serum. Starting with 1:2, prepare a twofold serial dilution of the Reactive (4+) serum control in PBS. Test each dilution using the procedure in paragraph d below to detect the greatest dilution that still produces minimal, but readily detectable, fluorescence. This dilution is used as a reading standard for the test.

(3) Reconstitution of Fluorescein-labeled Anti-human Globulin (Conjugate). The lyophilized conjugate is initially reconstituted (usually with water) according to the manufacturer's instructions. The reconstituted conjugate may be dispensed in small (never than 0.3 ml) aliquots and frozen at -20°C or lower. Before use, the conjugate is diluted with PBS containing 2 percent Tween 80 to the "working dilution" indicated by the manufacturer. NOTE: This "working dilution" should be checked by the using laboratory if any results are in doubt. This is done by testing a twofold serial dilution of conjugate that includes the "working dilution" indicated by the manufacturer against a Reactive (4+) control serum. The "working dilution" is defined as one tube (one twofold dilution) less than the greatest conjugate dilution to give a 4+ reaction. For example, if a 1:200 dilution of conjugate is the greatest dilution to give a 4+ reaction, a "working dilution" of 1:100 should be used.

(4) Preparation of Antigen Smears:

(a) Make two circles (1 cm inside diameter) on a clean slide with the diamond-point pencil. Circles should be separated as far as possible, but close enough together so that both may be covered completely by the 22 × 40 mm cover glass. Remove loose glass particles.

(b) Resuspend FTA-ABS antigen according to the manufacturer's instructions. Mix by aspiration with a capillary pipet at least 10 times. Check an aliquot by darkfield microscopy for even dispersion of the treponemes. Remix, if needed.

(c) Using the bacteriological loop, spread a loop of antigen in each circle on the slide.

(d) Air dry the slides at least 15 minutes.

(e) Fix smears in acetone for 10 minutes. Do not fix more than 60 slides/200 ml of acetone.

(f) Air dry the slides thoroughly.

(g) Store the acetone-fixed smears at -20°C or lower. These smears should not be thawed and refrozen, but they may be thawed and used as long as the expected reactions with control sera are produced.

(h) Allow frozen smears to warm to room temperature before testing.

d. Procedure:

(1) Set up each control and patient's serum (previously heated) in properly labeled test tubes according to the scheme provided in table 6-4. Add each control or patient's serum to 0.2 ml of the appropriate diluent (PBS or sorbent) using the bottom 0.05 ml of a 0.2 ml serological pipet. Mix the serum-diluent mixture by aspiration at least eight times and leave the pipet in the mixture. NOTE: The acceptable reaction for each control is also indicated in table 6-3. ALL controls must give these acceptable reactions before the results on patients' sera can be reported.

(2) Label antigen smears on the frosted end with a lead pencil. Preferably, each control and patient's serum should be run in duplicate (that is, use one slide for each fluid to be tested), if economically feasible.

(3) Carefully cover an entire antigen

Table 6-4. FTA-ABS Control Schema and Acceptable Reactions.

Table 6-4. FTA-ABS Control Schema and Acceptable Reactions.

	Serum	Diluent		Acceptable Reaction
		PBS	Sorbent	
Reactive Serum Controls				
1:5 in PBS	0.05 ml	0.2 ml	----	R(4+)
1:5 in Sorbent	0.05 ml	----	0.2 ml	R(4+ or 3+)
Minimally Reactive Reading Standard	0.05 ml	----	0.2 ml	R(1+)
Nonspecific Serum Controls				
1:5 in PBS	0.05 ml	0.2 ml	----	R(2+ to 4+)
1:5 in Sorbent	0.05 ml	----	0.2 ml	N
Nonspecific Staining Controls				
Due to PBS	----	0.2 ml	----	N
Due to Sorbent	----	----	0.2 ml	N
Patient's Serum	0.05 ml	----	0.2 ml	*

* Reported only if ALL controls are acceptable.

smear circle with approximately 0.03 ml of each dilution or control listed in table 6-3. The fluid may be spread by carefully rotating the tip of the pipet WITHOUT touching the antigen smear. Do not scratch the smear.

(4) Place the slide in a moist chamber with the reactants uppermost.

(5) Incubate the slides (in the moist chamber) at 35°C-37°C for 30 minutes.

(6) Following incubation, rinse the reactants from the slide with running PBS.

(7) Place all slides in slide carriers and soak in two changes of PBS for a total of 10 minutes (5 minutes each). Agitate the slides by dipping about 10 times during each change of PBS.

(8) Rinse the slides briefly in distilled water.

(9) GENTLY blot the slides dry with bibulous paper. Do not scratch the smears.

(10) Prepare the "working dilution" of conjugate with PBS containing 2 percent Tween 80. Spread 0.03 ml of "working dilution" of conjugate over each antigen circle.

(11) Replace the slides in the moist chamber and incubate them at 35°C-37°C for 30 minutes.

(12) After incubation, repeat the rinsing and GENTLE blotting as in steps 6-9 above.

(13) Add a SMALL drop of the PBS-glycerine mounting medium to each antigen circle and cover slip the slides.

(14) Assemble and align the fluorescent microscope and UV light source according to the manufacturer's instructions. Start the UV light and allow a warmup period as indicated by the manufacturer.

(15) Examine the slides by fluorescent microscopy using a darkfield condenser and 450× magnification (high-dry objective). Slides may be stored in the dark for about 4 hours before reading.

(a) Exciter Filters. Either a BG 12 or AO 702 filter may be used.

(b) Barrier Filters. Several types of barrier filters have been used. Some of these are OG 1, AO 724, AO 1124, B and L Y-8, and Zeiss 50/- (II/0).

Table 6-5. Reporting Results of FTA-ABS Tests.

Table 6-5. Reporting Results of FTA-ABS Tests.

Reading	Report
2+ to 4+	Reactive (R)
1+	Reactive (R)*
< 1+	Borderline (B)*
-	Nonreactive (N)

* Repeat all 1+ and < 1+ readings before reporting the results. A second serum should be tested on all Borderline results.

(16) Examine all Nonreactive smears by normal darkfield microscopy to confirm the presence of treponemes. Each smear should have about 30 treponemes/high-dry field.

(17) Compare all readings with the Minimally Reactive (1+) serum control.

e. Reporting Results. Before any results can be reported, all controls listed in table 6-3 must give acceptable reactions. If all are acceptable, report the results according to table 6-5 with the following exceptions:

(1) All 1+ and Borderline reactions should be retested before reporting results.

(2) A second serum should be requested on all cases identified as Borderline. Additional testing is probably not indicated if this second is also Borderline. Each patient's historical and physical findings will have to be carefully analyzed in these cases.

f. Sources of Error:

(1) The interpretation of fluorescence of the treponemes is subjective and only experience with the microscope will build confidence. Acceptable reactions on ALL controls must be achieved for valid results.

(2) Scratches in the antigen smear may produce confusing fluorescence.

(3) The "working dilution" of the conjugate indicated by the manufacturer should be checked to compensate for different equipment and procedures.

(4) Bacterial contamination of sera and conjugate may destroy the antibodies.

(5) Conjugate should not be frozen and thawed more than once. Freeze small aliquots and thaw only as much as required for a test run.

(6) Antigen smears should not be thawed and refrozen.

(7) Check all nonreactive smears by normal darkfield microscopy to verify the presence of treponemes.

g. Discussion:

(1) False-positive reactions have been reported in cases of lupus erythematosus (L.E.) in this test. Many of these demonstrate a peculiar "beaded" fluorescence of the treponemes. Other false positives may be detected as the test is studied more.

(2) Other treponemal diseases (pinta, bejel, and yaws) should be expected to give reactive results.

(3) FTA-ABS antibodies usually appear in the serum during the primary stage of syphilis and persist for life.

(4) Even after treatment the test usually remains reactive for life while most other serologic tests for syphilis become nonreactive, especially if treatment occurs before the tertiary stage of the disease.

(5) The FTA-ABS test is especially useful in the diagnosis of tertiary syphilis because the reagin tests may be nonreactive in about one-fourth of these cases.

SECTION C—TESTS ON SPINAL FLUID

6-9. VDRL Slide Test on Spinal Fluid:

a. Principle. VDRL slide test antigen is made more sensitive by adding an equal volume of 10-percent saline. A drop (1/100 ml) of this sensitized antigen is added 0.05 ml of unheated spinal fluid in a 16 mm diameter by 1.75 mm deep concavity of an agglutination slide. The slide is rotated for 8 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100× magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the spinal fluid.

b. Reagents and Equipment:

(1) Patient's spinal fluid, unheated.

(2) Reactive, minimally Reactive and Nonreactive control sera, diluted at least 1:80 before testing.

(3) VDRL (Cardiolipin Microflocculation) slide test antigen.

(4) VDRL buffered saline, pH 6.0 ± 0.1.

(5) Saline, 0.9%.

(6) Saline, 10%. Q.s. 10 g of NaCl to 100 ml with distilled water.

(7) Pipets, serological, 0.2 ml.

(8) Slide, agglutination, with 16 mm diameter by 1.75 mm deep concavities.

(9) Bottle, 30 ml, round, glass-stoppered, with flat innerbottom, the same as used for VDRL tests on serum.

(10) Slide rotator, set at 180 rpm.

(11) Syringe, 1 ml.

(12) Needle, hypodermic, 22-gage with bevel removed, pretested to deliver 1/100 ml/drop.

c. Preliminary Steps:

(1) Preparation of Spinal Fluid. Usually the third tube of spinal fluid collected is reserved for serologic tests. The fluid should be free of hemolysis and cells. Centrifuge the specimen and test the clear supernate.

(2) Preparation of Sensitized VDRL Antigen Emulsion:

(a) Prepare a batch of VDRL slide test antigen emulsion according to instructions in paragraph 6-5b (2).

(b) Within 2 hours of testing time, mix 1 part of freshly prepared VDRL antigen emulsion with 1 part of 10-percent saline. Mix well and let stand at least 5 minutes, but use the sensitized antigen in less than 2 hours.

(3) Testing of Delivery Needle:

(a) Gently mix the sensitized antigen emulsion and fill a 1-ml syringe fitted with a 22-gage needle with the bevel removed.

(b) Holding the needle vertically, determine the number of drops/milliliter. This test requires 100 drops ± 2 drops/milliliter.

(c) Adjust the bore of the needle until 100 drops/ml are delivered.

(4) Preparation of Control Sera. Controls for this test are prepared from unheated serum, NOT spinal fluid. The Reactive serum used as a control must have a titer of 1:80 or greater when tested by the VDRL test for spinal fluid. Test the Reactive serum after it has been frozen for several days.

(3) Starting at 1:80, prepare a twofold serial dilution of unheated Reactive serum in 0.9-percent saline.

(b) Test each dilution by the VDRL test for spinal fluid and record dilutions that give Reactive, minimally Reactive, and Nonreactive results. Use these three dilutions as controls each time the test is performed.

d. Procedure:

(1) Allow all reagents to warm to room temperature.

(2) Pipet 0.05 ml of clear, unheated spinal fluid to a concavity in the agglutination slide.

(3) Pipet 0.05 ml of each diluted control serum to the slide.

(4) Add one drop (1/100 ml) of sensitized VDRL antigen emulsion to each control or spinal fluid with a pretested 22-gage needle.

(5) Rotate the slide for 8 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(6) Read the tests at 100× magnification (10 × ocular and 10 × objective) for flocculation of the antigen particles.

e. Reporting Results: Before any results can be reported, the control sera must produce the expected reactions. If the controls indicate valid results, report the patient's spinal fluid as either Reactive or Nonreactive, as appropriate. NOTE: The Weakly Reactive report is not used for this test. Any reactivity is significant.

f. Sources of Error:

(1) Be sure that the VDRL antigen emulsion is sensitized by adding equal parts of 10-percent saline.

(2) The antigen drop (1/100 ml/drop) must be accurate within ±2 drops/milliliter.

(3) Reactive control sera must have a titer of at least 1:80 when tested by the spinal fluid test.

(4) Spinal fluids containing gross blood or microbial contamination are unsatisfactory.

(5) All reagents must be at room temperature for testing.

g. Discussion:

(1) False positives rarely occur in tests for syphilis performed on spinal fluid. A Reactive test usually indicates present or past neurosyphilis, especially if the cell count and protein content of the spinal fluid are elevated.

(2) The spinal fluid must be Nonreactive in latent syphilis. A Reactive spinal fluid indicates that the disease has progressed to the tertiary stage.

(3) The spinal fluid is Reactive in about one fourth of cases in the secondary stage of syphilis, thus indicating that central nervous system involvement has begun.

6-10. Colloidal Gold Test on Spinal Fluid. The colloidal gold test on spinal fluid was widely used in the past for the differentiation of the various types of neurosyphilis and other central nervous system conditions. In the test, a colloidal suspension of gold is added to serially diluted spinal fluid. Abnormal spinal fluid proteins cause partial to complete precipitation of the gold with a variety of color changes. More recently, this test was mainly used in the study of multiple sclerosis, but the test is being rapidly replaced by the simpler, and more reproducible, gum mastic and benzoin tests.

SECTION D—OTHER TESTS FOR SYPHILIS

6-11. Reiter Protein Complement Fixation (RPCF) Test. This test is the Kolmer complement fixation method using an extract of the Reiter strain of treponeme as the antigen. In the test, complement is "fixed" during the reaction of the Reiter protein and treponemal antibodies in the syphilitic patient's serum. Complement is, therefore, no longer available when the second antigen-antibody system, sheep erythrocytes and hemolysin, is added—hemolysis cannot occur. The complexity and relative insensitivity of the test originally led to the test being essentially replaced by the TPI test described in the next paragraph. More recently, the FTA-ABS test has replaced nearly all other treponemal tests for syphilis.

6-12. Treponema Pallidum Immobilization (TPI) Test. The TPI test detects treponemal antibodies that immobilize living Nichols strain of *T. pallidum* in the presence of complement. The requirement for live treponemes from rabbits' testes severely limits the practicality of this test for the average laboratory. Although the test is highly specific for treponemal diseases, it has been essentially replaced by the FTA-ABS test. Unlike the FTA-ABS test, the TPI test frequently becomes negative following treatment of the disease.

6-13. Automated Reagin Tests. Attempts to automate tests for syphilis have mainly involved the use of the RPR carbon-containing antigens and various AutoAnalyzer components (Technicon Instruments Corp.). Most recent applications have involved continuous filtering of the reactants on a moving strip of paper with visual interpretation of the flocculated carbon particles. Test speeds of up to 100/hr have been reported with results equivalent to manually performed tests.

Chapter 7

TESTS FOR INFECTIOUS MONONUCLEOSIS

7-1. Introduction. Infectious mononucleosis is an acute disease, characterized by fever, headache, sore throat, enlarged lymph nodes, splenomegaly, malaise, and mild hepatitis. It is believed to be caused by the Epstein-Barr virus (or some similar herpeslike virus) with transmission most likely by intimate oral contact. Young adults (16-25 years old) are most frequently affected; consequently, infectious mononucleosis is a significant problem for the military services.

a. No known treatment is available for infectious mononucleosis, but bed rest usually resolves overt illness in 2 to 4 weeks with only rare fatalities or permanent disabilities. Aside from lost time from the job, the disease is mainly important because the symptoms are shared by certain severe, but treatable, diseases. The disease has been most frequently misdiagnosed initially as diphtheria or bacterial tonsillitis. In addition, the disease must be differentiated from viral hepatitis and several other diseases. An accurate differential diagnosis of infectious mononucleosis is based primarily on laboratory studies along with the typical clinical observations.

b. One of the first laboratory findings in infectious mononucleosis concerns the white blood cells. The white blood cell count is usually over 10,000 per cmm with a differential count of over 50 percent lymphocytes. In addition, atypical lymphocytes are present. The characteristics of these lymphocytes and atypical lymphocytes can be found in the hematology manual. However, because lymphocytosis and atypical lymphocytes can also be found in other diseases, serologic tests are also necessary for the diagnosis of infectious mononucleosis.

c. The serodiagnosis of infectious mononucleosis is another good example of the use of cross-reactivity in serology. The majority of patients with the disease eventually produce antibodies (agglutinins) that agglutinate sheep and horse erythrocytes. Since these erythrocytes have no logical relationship to the causative organism of the disease, this is another example of cross-reactivity—shared antigens among different species. Unfortunately, the diagnosis of infectious mononucleosis is complicated by the fact that several microorganisms, as well as plants and animals, possess antigens that also can stimulate the production of sheep (and horse) erythrocyte agglutinins. Collectively, these antigens are called heterophile antigens and the antibodies—heterophile antibodies. The serodiagnosis of infectious monucleosis entails the detection of these heterophile antibodies along with the differentiation of the antibodies due to infectious mononucleosis from other heterophile antibodies that might be present in the serum. Since these heterophile tests are relatively simple, specific serologic tests using the virus probably never will become practical.

d. Serotests for heterophile antibodies can be divided into two categories: presumptive and differential tests.

(1) Presumptive heterophile tests. These are screening tests that utilize sheep erythrocytes (more recently, horse erythrocytes) as the antigen to detect the presence of heterophile antibodies. Several commercially available slide test kits have been developed. Many of these slide tests also include reagents to identify (differentiate) the particular heterophile antibody present.

(2) Differential heterophile tests. Most differential tests are based on the differential absorption of the various types of heterophile antibodies from the serum by suspensions of guinea pig kidneys (GPK) and beef (ox) erythrocytes (BE). One type of heterophile antibodies is adsorbed by GPK only, another by BE only, and the third by both GPK and BE. Other differential heterophile tests are based on the use of enzyme-treated sheep erythrocytes; while still another test detects ox erythrocyte hemolysins that are produced in response to infectious mononucleosis but not in other conditions that produce heterophile antibodies. One of these differential tests must usually be performed each time heterophile antibodies are detected.

7-2. Significance of Heterophile Antibodies. As with any antigenic stimulation, heterophile antigens must first gain entry into the body before the RE system can respond by producing antibodies. For example, heterophile antigens, such as certain gram-negative bacilli of the intestinal tract, may enter through minor lesions of the intestinal wall. Or a heterophile antigen may enter the body by setting up an infection; such is the case with infectious monucleosis. In addition, heterophile antigens can be introduced by parenteral infections of horse serum. This is a rare occurrence today, but in the recent past, several diseases, such as pneumonia and diphtheria, were treated with antisera produced in horses. Regardless of the route of entry of the heterophile antigens, all stimulate the production of sheep erythrocyte agglutinins (heterophile antibodies). Fortunately, despite the multitude of heterophile antigens that man may be exposed to, heterophile antibodies can be conveniently differentiated by absorption studies and other methods into only three main types—Forssman, serum sickness, and infectious mononucleosis types. This detection and differentiation of the three types of heterophile antibodies is the responsibility of the clinical serologist.

a. Forssman (Native) Antibodies. The heterophile antibodies that are produced in response to incidental contact with heterophile antigens, such as certain *Salmonella*, *Shigella*, and other bacterial species as well as several other antigens of plant or animal origin, are called Forssman or native antibodies. These antibodies are found in low titers, in nearly all normal adults. The antibodies have no clinical significance, except that they must be differentiated from other types of heterophile antibodies. The differentiation is accomplished by absorbing the patient's serum (antibodies) with suspensions of guinea pig kidney (GPK) and beef (ox) erythrocytes (BE). Forssman antibodies are absorbed from the serum by the GPK, but not by the BE. In other words, after absorption with GPK, the serum no longer agglutinates sheep (or horse) erythrocytes; while serum absorbed with BE still agglutinates sheep erythrocytes. The other two types of heterophile antibodies have different absorption patterns following treatment of serum with GPK and BE.

b. Serum Sickness Antibodies. These sheep erythrocyte agglutinins are stimulated in man in response to the injection of horse serum or products from horse serum. This type of heterophile antibody will rarely be encountered today due to the declining use of horse serum injections. If present, they may be differentiated from other heterophile antibodies by the fact that they are absorbed from the serum by BOTH guinea pig kidney (GPK) and beef erythrocytes (BE). Of course, a history of horse serum injections is significant too.

c. Infectious Mononucleosis Antibodies. Heterophile antibodies due to infectious mononucleosis first appear in the serum of a low percentage of patients during the first week of illness. The precentage increases until the majority of cases have detectable titers by the fourth week, but the titers do not parallel the severity of the disease. These titers start to drop rapidly after about the fourth week and rarely persist for more than a few months. The peak titers of these antibodies are usually much higher than seen with Forssman and serum sickness, but usually the differential tests with GPK and BE still must be performed to specifically identify the source of the antibodies. Infectious mononucleosis antibodies are absorbed by BE, but not by GPK—just the opposite pattern of Forssman antibodies.

7-3. Slide Screening Test for Heterophile Antibodies:

a. Principle. Patient's serum is mixed with a suspension of sheep erythrocytes on a slide. Agglutination of the erythrocytes indicates the presence in the serum of unspecified heterophile antibodies.

b. Reagents and Equipment:

(1) Patient's serum, fresh or inactivated.

(2) Positive and negative control sera (collected from previous patients or available commercially).

(3) Sheep erythrocytes, washed, 5 percent suspension in saline. NOTE: These cells should be at least 24 hours, but less than 1 week, old. Defibrinated sheep blood for bacteriologic media may be used if the cells are of the proper age.

(4) Saline, 0.9%.

(5) Blood collecting equipment (see paragraph 3-2).

(6) Slide rotator, set at 120 rpm.

(7) Microscope slides, glass.

(8) Applicator sticks or toothpicks.

c. Preliminary Steps:

(1) Collect the patient's serum.

(2) Wash sheep erythrocytes with saline and prepare a 5 percent suspension of cells in saline as described in chapter 4, paragraph 4-2.

(3) Thaw aliquots of positive and negative control sera. Mix the sera gently and allow them to warm to room temperature.

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(4) Label slides for positive control, negative control, and patient's sera.

d. Procedure:

(1) Place one drop of each serum to be tested (including controls) on appropriately labeled slides.

(2) Add one drop of a 5 percent suspension of sheep erythrocytes to each serum drop, and mix each throughly.

(3) Rotate the slide for 2 minutes at 120 rpm.

(4) IMMEDIATELY examine the mixtures for macroscopic agglutination of the erythrocytes.

e. Reporting Results. If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Sheep Erythrocyte Screening Test: Negative.

(2) Positive results (agglutination) should be confirmed by the Paul-Bunnell presumptive test (see paragraph 7-4) or one of the commercial kits. Then, if indicated, the Davidsohn differential heterophile test described in paragraph 7-5 or one of the commercially available differential tests should be performed.

f. Sources of Error:

(1) Drying of the mixture may be confused with agglutination.

(2) Certain lots of sheep cells may be either hypersensitive or insensitive to heterophile antibodies. Use of positive and negative control sera will detect these problems. Cell suspensions should be used only on the day they are prepared.

(3) A delay in reading the test after the rotation period may result in false positives due to delayed agglutination or drying.

(4) All reagents and sera must be used at room temperature. Cold reagents may result in delayed reactions. In addition, sera containing cold agglutinins may cause agglutination.

(5) If indicated, all negative patients should be retested at weekly intervals to detect delayed antibody production.

g. Discussion:

(1) This test detects only the presence of heterophile antibodies. A differential test is needed to identify the antibody present.

(2) Several slide tests using horse erythrocytes have been developed. Several concentrations of cells are used. These tests are reportedly more sensitive than sheep cells and more specific for infectious mononucleosis. Several modifications are available commercially. Some of these are described briefly in paragraph 7-6.

7-4. Paul-Bunnell Presumptive Heterophile Test:

a. Principle. A suspension of sheep erythrocytes is added to serially diluted serum. Agglutination of the erythrocytes indicates the presence of unspecified heterophile antibodies.

b. Reagents and Equipment:

(1) Patient's serum, inactivated.

(2) Positive and negative control sera (collected from previous patients or available commercially).

(3) Sheep erythrocytes, washed, 2 percent suspension in saline. NOTE: These cells should be at least 24 hours, but less than 1 week, old. Defibrinated blood may be used.

(4) Saline, 0.9%.

(5) Blood collecting equipment (see paragraph 3-2).

(6) Test tubes, 12 × 75 mm.

(7) Pipets, serological, 0.25 and 1.0 ml.

(8) Water bath, 56°C.

c. Preliminary Steps:

(1) Thaw aliquots of positive and negative control sera. NOTE: Only 0.1 ml of each serum is needed for the test.

(2) Inactivate the patient's serum and both control sera at 56°C for 30 minutes.

(3) Wash sheep erythrocytes with saline and prepare a 2 percent suspension of cells in saline as described in chapter 4, paragraph 4-2.

d. Procedure:

(1) Place 10 test tubes in racks for each serum to be tested, including the control sera.

(2) With a 1.0 ml pipet, place 0.4 ml of saline in the first tube for each serum and 0.25 ml in the remaining tubes.

(3) With a 0.25 ml pipet, place 0.1 ml of INACTIVATED serum in the first tube. (NOTE: For greater accuracy, this pipet may be discarded at this point and a new pipet used for each tube.) Mix the contents of the tube by aspiration and transfer 0.25 ml of saline-serum mixture to the second tube. Repeat this through the ninth tube from which 0.25 ml of saline-serum mixture is discarded.

(a) The tenth tube is an antigen control and receives no serum. Only one of these needs to be prepared for each batch of tests.

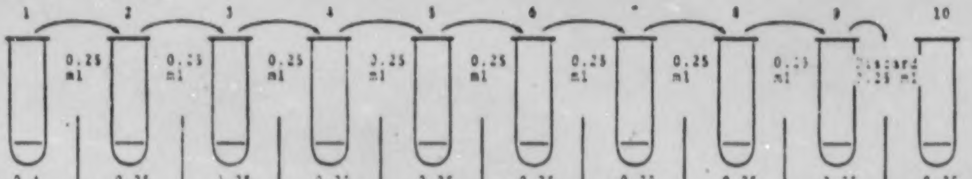
(b) The serial dilution prepared in this manner is a twofold dilution starting with 1:5 and ending with 1:1280 in the ninth tube. These dilutions will be increased when the antigen suspension is added.

(4) Examine the tubes. Each should contain 0.25 ml of fluid if the dilution has been performed properly.

(5) Add 0.1 ml of a 2 percent suspension

Table 7-1. Paul-Bunnell Presumptive Heterophile Test.

Table 7-1 Paul-Bunnell Presumptive Heterophile Test



Tube Number	1	2	3	4	5	6	7	8	9	10
Saline, ml	0.4	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
		0.25 milliliters of:								
Serum, ml	0.1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	None (Control)
Dilution After Serum Transfer	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	---
Sheep Erythrocytes, ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total Volume, ml	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Final Dilution of Serum	1.7	1:14	1:28	1:56	1:112	1:224	1:448	1:896	1:1792	---

of sheep erythrocyte to each tube. This makes the final serum dilution in the first tube 1:7, the second 1:14, etc.

(6) Shake the rack to mix the contents of the tubes and let them stand undisturbed at ROOM TEMPERATURE for 2 hours.

(7) At the end of 2 hours, examine the tubes for agglutination by gently shaking the tube and comparing each with the cell control (tube #10). The tubes may be examined with the aid of a hand lens or microscope mirror as shown in chapter 5, figure 5-1. Record the highest serum dilution showing definite agglutination. The Paul-Bunnelll presumptive test is shown graphically in table 7-1.

e. Reporting Results. If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Less than 1:7. This is the lowest serum dilution actually tested.

(2) In positive tests, the highest serum dilution to produce agglutination is reported as the titer. NOTE: The serum dilution INCLUDES the dilution due to the antigen. Usually positive results will have to be tested by one of the differential heterophile tests. See paragraph 7-4g for a discussion of the signficance of test titers.

f. Sources of Error:

(1) Certain lots of sheep cells may be hypersensitive or insensitive to heterophile antibodies. Use of positive and negative control sera will detect these problems. Cell suspensions should be used only on the day they are prepared.

(2) Unless the serum complement is inactivated, hemolysis may destroy the cells and obscure agglutination. Not all sera will contain sheep erythrocyte hemolysins, but the complement must be inactivated in all sera to avoid confusion.

(3) All sera and reagents must be used at room temperature. Cold reagents may result in delayed reactions. In addition, sera containing cold agglutinins may cause agglutination.

(4) Prozone reactions may occur with this test. For a discussion of prozone reactions see chapter 2, paragraph 2-14.

(5) If indicated, all negative patients should be retested at weekly intervals to detect delayed antibody production, because not all patients will have detectable antibody levels when first tested.

g. Discussion:

(1) Although titers of infectious mononucleosis antibodies are usually higher than titers of other heterophile antibodies, titers have no relation to the severity of the disease. Liver function tests are generally more reliable for following the course of the disease so that the presumptive heterophile titer is used mainly as a basis for the performance of the differential heterophile test.

(2) Titers of 1:56 or less are considered negative for infectious mononucleosis in the absence of clinical and hematologic findings.

(3) Titers of 1:224 or greater are considered to be presumptive evidence of infectious mononucleosis if clinical and hematologic signs are present. In addition, the patient must not have had recent injections of horse

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serum. If these conditions are met, no further testing is required.

(4) A differential heterophile test must be performed in the following situations:

(a) A presumptive titer of LESS than 1:224, but the patient has signs of infectious mononucleosis.

(b) A presumptive titer of GREATER than 1:224, but the patient has NO signs of infectious mononucleosis.

(c) A presumptive titer of 1:224 or greater, and the patient has a history of horse serum injections.

7-5. Davidsohn and Differential Heterophile Test:

a. Principle. When indicated by the findings of the presumptive test (see paragraph 7-4g), separate aliquots of the patient's serum are absorbed with suspensions of guinea pig kidneys (GPK) and beef erythrocytes (BE). After centrifugation to remove the GPK and BE, the supernate from each antigen is retested by the presumptive test. A drop in titer of more than three tubes indicates absorption of the antibody by the given antigen. The absorption patterns for the three types of heterophile antibodies are summarized in table 7-2.

b. Reagents and Equipment:

(1) Guinea pig kidney antigen, available commercially.

(2) Beef erythrocyte antigen, available commercially.

(3) Control sera, various types are available commercially.

(4) Centrifuge.

(5) For other items, see paragraph 7-4b.

c. Preliminary Steps:

(1) Determine from the presumptive titer and clinical data if the differential test is indicated. Record the presumptive titer for future reference.

(2) Inactivate all sera at 56°C for 30 minutes. NOTE: Only 0.4 ml of each serum will be needed for the test.

(3) Reconstitute the GPK and BE antigens according to the manufacturer's instructions.

(4) Unless the GPK and BE manufacturer's instructions use different times and volumes, absorb the patient's serum (and each control serum) as follows:

(a) GENTLY, but thoroughly, mix the GPK and BE suspensions.

(b) Pipet 1.0 ml of GPK and 1.0 ml of BE to tubes labeled "GPK" and "BE", respectively.

(c) Add 0.2 ml of INACTIVATED serum to each tube and mix GENTLY by tapping the tube.

(b) Let the mixtures stand at ROOM TEMPERATURE for at least 3 minutes, with gentle shaking at intervals.

(e) At the end of the incubation period, centrifuge both tubes at 1500 rpm for 10 minutes.

(f) Carefully, collect at least 0.5 ml of supernate from each tube and perform the presumptive test on each supernate. NOTE: The supernate already represents a serum dilution of 1:5.

d. Procedure:

(1) Place two rows of 10 tubes each in racks for each serum to be tested. Label one row "GPK" and the other "BE".

(2) Starting with the SECOND tube of each row, pipet 0.25 ml of saline into each tube. The first tube receives no saline.

(3) With a 0.25 ml pipet, place 0.25 ml of GPK supernate in the first and second tubes of the row labeled "GPK". Pipet 0.25 ml of BE supernate into the first and second tubes of the row labeled "BE".

(a) The serum in the first tube needs no additional dilution, because the dilution in the supernate is already 1:5.

(b) For greater accuracy, the pipets may be discarded at this point and a new pipet used for each tube.

(4) Mix the contents of the SECOND tube by aspiration and transfer 0.25 ml of supernate-saline mixture to the third tube. Repeat this through the ninth tube from which 0.25 ml of supernate-saline mixture is discarded. The tenth tube is the antigen (sheep cell) control and receives no supernate.

(5) Repeat step #4 until all supernates are diluted.

(6) Examine the tubes. Each should contain 0.25 ml of fluid if the dilution has been performed properly.

(7) Add 0.1 ml of a 2 percent suspension of sheep erythrocytes to each tube. This makes the final serum dilution in the first

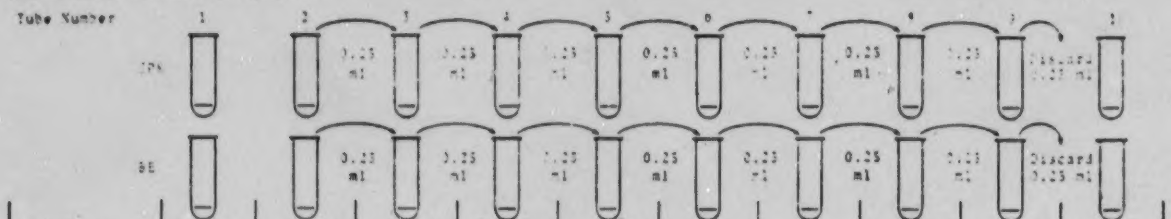
Table 7-2 Absorption Patterns of Heterophile Antibodies.

Types of heterophile antibody	Absorbed by guinea pig kidney antigen	Absorbed by beef erythrocyte antigen
Forssman (natural)	[illegible]	[illegible]
Serum sickness	[illegible]	[illegible]
Infectious mononucleosis	[illegible]	[illegible]

Table 7-2. Absorption Patterns of Heterophile Antibodies.

Table 7-3. Davidsohn Differential Heterophile Test.

Table 7-3. Davidsohn Differential Heterophile Test.



Saline, ml	None	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Supernatant Fluid (1:5 dilution of serum)	0.25	0.25	----	----	----	----	----	----	----	----
Serum Transfer, ml	----	----	0.25 milliliters of:							None (Control)
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	
Dilution After Serum Transfer	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	----
Sheep Erythrocytes, ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total Volume, ml	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Final Dilution of Serum	1:7	1:14	1:28	1:56	1:112	1:224	1:448	1:896	1:1792	----

tube 1:7, the second 1:14, etc.—the same as in the presumptive test.

(8) Shake the racks to mix the contents and let them stand undisturbed at ROOM TEMPERATURE for 2 hours.

(9) At the end of 2 hours, record the titer of each supernate. The performance of the titration part of the Davidsohn differential heterophile test is shown graphically in table 7-3.

e. Reporting Results. Before any results can be reported, the control sera must yield the expected results. If they do, proceed as follows:

Table 7-4. Example, Results of Differential Heterophile Tests.

Presumptive test titer	Differential test after adsorption with--		Interpretation with regard to infectious mononucleosis
	Guinea pig kidney	Beef red cells	
1:224	1:112	1:7	POSITIVE
1:224	1:56	0	POSITIVE
1:224	1:28	0	POSITIVE
1:224	1:14	1:112	NEGATIVE
1:224	1:7	1:112	NEGATIVE
1:56	1:56	0	POSITIVE
1:56	1:28	0	POSITIVE
1:56	1:14	0	POSITIVE
1:56	1:7	0	POSITIVE
1:28	1:28	0	POSITIVE
1:28	1:14	0	POSITIVE

Table 7-4. Example, Results of Differential Heterophile Tests.

(1) Record the presumptive heterophile titer, as well as the titers of the supernates following absorption with GPK and BE, on the patient's laboratory slip.

(2) Compare the titers of the supernates from the GPK and BE absorption with the presumptive titer. Significant absorption by the GPK and/or BE is indicated by a drop in titer of MORE than three tubes when compared with the presumptive titer. Use table 7-2 to determine the heterophile antibody present. Examples of differential test findings are provided in table 7-4.

(3) Add a statement to the patient's laboratory slip, such as "positive for infectious mononucleosis antibodies" or some appropriate comment.

f. Sources of Error:

(1) The sources of error listed for the presumptive heterophile test (see paragraph 7-4f) also apply to this test.

(2) The manufacturer's instructions must be followed scrupulously for the reconstitution and use of the GPK and BE antigens.

g. Discussion:

(1) Occasionally, GPK and BE absorption patterns will not fit the three categories listed in table 7-2.

(2) Several commercially available slide tests have been developed that incorporate presumptive and differential features into the same test. Some of these tests are based on GPK and BE absorption but offer the advantage of speed—a critical commodity in

the clinical laboratory. These tests are rapidly making the Paul-Bunnell and Davidsohn tests impractical.

(3) Due to the declining use of horse serum infections, the differential heterophile test could actually be adequately performed using the GPK antigen only. However, the use of the BE antigen does serve as a double check on the GPK results.

7-6. Miscellaneous Heterophile Tests. Several other tests for infectious mononucleosis are in use. Most of these are modifications of the basic Paul-Bunnel presumptive and the Davidsohn differential tests; a few are based on different principles. The principle of several of these tests will be described briefly and one or two examples given when commercial products are available. In all cases, be sure to adhere to the manufacturer's instructions in performing these tests.

a. Ox Hemolysin Test. In this test ox (beef) erythrocytes and complement are added to serially diluted serum. Patients with infectious mononucleosis produce higher titers of ox hemolysins than normal individuals. Various modifications of this test are in use, but titers of more than 1:40 to 1:48 are usually considered indicative of infectious mononucleosis. This test has been found to be quite specific and sensitive, but time-consuming.

b. Tests with Papain-treated Sheep Erythrocytes. The proteolytic enzyme, papain, has been found to destroy the receptor sites for infectious mononucleosis antibodies on sheep erythrocytes. Serum is tested against both papainized and untreated sheep cells. In infectious mononucleosis, the untreated cells agglutinate, but the papainized cells do not, or else their agglutination is delayed when compared to the untreated cells. Examples of commercial procedures using this principle are the Bacto-Hetrol Slide Test (Difco) and MonoStat (COLAB).

c Modified Presumptive Tests Using Horse Erythrocytes. Horse erythrocytes have been found to be more sensitive with fewer false positives than sheep erythrocytes for the detection of infectious mononucleosis antibodies. Both formalinized cells and cells preserved in 3.8% citrate give acceptable results. Several concentrations of cells have been tested. In addition, when properly stored, these cells are stable for up to 1 year. Examples of slide tests using formalinized horse erythrocytes are the I.M. Kit (Microbiological Research Corporation) and Mono-Test (Wampole).

d. Modified Davidsohn Differential Tests. A number of slide modifications of the basic GPK and BE absorption test are available commercially. Since absorption tests seem to be the most widely accepted method for the differential identification of heterophile antibodies, these slide tests offer a distinct advantage—speed—over the original tube test. In addition, these tests delete the need for a presumptive test. An example of a slide differential heterophile test that uses the traditional sheep cells as the indicator antigen is the Monosticon (Organon, Inc.) test. Another test kit from Organon, the Monosticon Dri-Dot, consists of separate spots of dried GPK and horse erythrocytes on a paper card. The serum is added to the GPK. Then, this mixture is stirred into the horse erythrocytes. Another test kit that uses horse erythrocytes, but in suspension form, along with both GPK and BE suspensions is the Monospot Slide Test (Ortho). An important consideration in using any of these tests is to be sure to follow the manufacturer's instructions at all times. Although some of these manufacturers include procedures for titrating the antibodies, titrations would not appear to be economically feasible in view of the fact that the titer does not parallel the severity of the disease. Liver function tests are considered to be better than titrations for following the course of the disease.

7-7. Interpretation of Results. In making a diagnosis of infectious mononucleosis, the physician must consider the hematologic, clinical, and serologic findings, as well as the history of the patient. Of these, the serologic findings provide the most specific evidence of disease— expecially if one of the differential heterophile tests is used. These tests are needed to identify the antibody present.

a. Forssman (Native). Antibodies. These are usually present in low titer in most normal adults. They are absorbed from the serum by GPK and not by BE suspensions. Forssman antibodies have no clinical significance except that they must be differentiated from other heterophile antibodies.

b. Serum Sickness Antibodies. These antibodies may reach relatively high titers. As the use of injections or horse serum products has declined, these antibodies have become more rare. If present, they are absorbed by both GPK and BE suspensions.

c. Infectious Mononucleosis Antibodies. These antibodies may reach very high titers during the disease, but not all patients produce detectable levels at the same point in the disease. A presumptive titer of 1:224 or greater with sheep erythrocytes is considered evidence of disease when hematologic, clinical, and historical findings are consistent with infectious mononucleosis. At other times, a differential heterophile test may be required. These antibodies are absorbed by BE but not GPK suspensions. The presence of ox hemolysins and the inability of these antibodies to agglutinate papainized sheep erythrocytes can also be used to identify the disease as infectious mononucleosis.

Chapter 11

TESTS FOR RHEUMATOID ARTHRITIS

11-1. Significance of Rheumatoid Factors:

a. The Disease. Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology affecting the joints and synovial membranes. Early symptoms of joint pain and muscular stiffness may occur at any age with females more frequently affected than males. These early symptoms are not unique to rheumatoid arthritis so several other diseases must be ruled out in the differential diagnosis. Later in the disease, subcutaneous nodules are frequently noted over bony prominences. The disease may ultimately progress to severe skeletal deformity and complete immobilization of the affected joints.

b. Rheumatoid Factors. Depending on the test method used, about 80 percent of classical cases of rheumatoid arthritis have anti-gamma-globulin antibodies in their serum. These antibodies are mainly against IgG and react with the gamma globulin from several species of animals, as well as from the patient. Because these antibodies are most frequently associated with rheumatoid arthritis, they are called rheumatoid factors or, simply, RF.

(1) Rheumatoid factors were originally thought to be a single macroglobulin related to IgM immunoglobulin, but they are now known to be, in fact, IgM immunoglobulin along with small quantities of IgG and IgA. The presence of rheumatoid factors in IgM, IgG, and IgA is further evidence that these factors are antibodies. In effect, they are antibodies against antibodies.

(2) Unfortunately, the mere presence of rheumatoid factors in the serum does not confirm a diagnosis of rheumatoid arthritis, because a varying percentage of several other diseases may produce detectable levels of so-called rheumatoid factors. In addition, a negative test for rheumatoid factors does not rule out the possibility of rheumatoid arthritis, because about 20 percent of actual cases have negative tests. The presence or absence of rheumatoid factors also varies with the age of the patient, with children less apt to produce the factors, while older individuals are more frequently positive—even in the absence of rheumatoid arthritis. Despite these variations in production, serotests for the detection of rheumatoid factors in the serum are useful in the diagnosis of rheumatoid arthritis. Detecting increases in the titer of these factors is especially significant.

c. Tests for Rheumatoid Factors. Several types of tests have been developed for the detection of rheumatoid factors in the serum. Some of these tests, such as those involving radioimmunoassay, are very complex and, therefore, will be impractical for the average clinical laboratory. Most of the practical tests are based on the passive agglutination of either erythrocytes or inert particles coated with gamma globulin from animals or humans. The erythrocytes most frequently used are either fresh or preserved sheep cells, while polystyrene latex particles are the most commonly used inert particles. In general, the latex tests are more sensitive (more false positives with other diseases) than the erythrocyte tests, but a positive erythrocyte test is more likely to indicate rheumatoid arthritis. Examples of both types of tests are included in this manual. Most of these tests are available commercially in kit form.

11-2. Latex Slide Screening Test:

a. Principle. A 1:20 dilution of patient's serum in glycine-saline buffer diluent is mixed on a slide with a suspension of globulin-coated polystyrene latex particles. Agglutination (macroscopic clumping) of the latex particles indicates the presence of rheumatoid factors in the patient's serum.

b. Reagents and Equipment. Available in kit form from several sources. NOTE: Be sure to follow the manufacturer's instructions.

(1) Patient's serum, fresh or frozen.
(2) Glycine-saline buffer diluent.
(3) Latex-globulin reagent.
(4) Control sera, positive and negative.
(5) Test tubes, 12 × 75 mm.
(6) Pipets, 1 ml serological and transfer.
(7) Glass slides.
(8) Applicator sticks or toothpicks.

c. Preliminary Steps. In a test tube, prepare an approximate 1:20 dilution of the patient's serum by adding 1 drop of serum to 1 ml of glycine-saline buffer diluent and mixing gently. Do NOT dilute the control sera. Frozen sera should be at room temperature before testing.

d. Procedure:

(1) Place 1 drop of diluted patient's serum on a section of the slide.

(2) Place 1 drop each of UNDILUTED positive and negative control sera on separate sections of the slide.

(3) Gently resuspend the latex-globulin reagent and add 1 drop to each serum.

(4) Using a separate applicator stick for each serum, mix each serum thoroughly with the latex-globulin reagent and spread each mixture over an area about the size of a quarter.

(5) Tilt the slide from side to side for 1 minute and observe for macroscopic clumping (agglutination) of the latex particles.

e. Reporting Results: If the positive and negative control sera give the expected results, report the results of the patients' sera as follows:

(1) No visible clumping—Negative.

(2) Partial clumping—Weakly Reactive or Weekly Positive.

(3) Complete clumping—Reactive or Positive.

f. Sources of Error:

(1) Store reagents and perform procedures according to manufacturers' instructions. Do not allow the latex-globulin reagent to freeze.

(2) All glassware must be scrupulously clean, because tests for rheumatoid factors are very sensitive to pH and ionic changes. Spontaneous agglutination can occur if the pH falls to the isoelectric point of the gamma globulin (about 6.6); therefore, a pH of 8.2 must be maintained.

(3) Failure to dilute the patient's serum before testing may result in false-negative results due to the prozone phenomenon. Some kit manufacturers recommend heat-inactivated serum (56°C for 30 min) to reduce the incidence of prozoning.

(4) Do not read the test after the time period specified by the manufacturer because drying of the reactants may resemble agglutination.

11-3. Eosin-Latex Slide Screening Test:

a. Principle. Patient's serum (or whole blood) is mixed with a drop of eosin solution on a slide. Then, a drop of a suspension of uncoated latex particles is mixed with the serum-eosin mixture. Spontaneous agglutination of the latex particles indicates the presence of rheumatoid factors in the serum. The addition of the eosin solution before the latex particles reduces the incidence of false-positive tests.

b. Reagents and Equipment. Available commercially in kit form.

(1) Patient's serum, fresh or frozen.

(2) Positive control serum.

(3) Eosin reagent.

(4) Latex suspension.

(5) Glass slides.

(6) Toothpicks or similar stirrers.

(7) Light source.

c. Preliminary Steps:

(1) When a new batch of reagents is first opened, check them for proper storage by mixing 1 drop of eosin reagent with 2 drops of latex reagent on a slide. Rock the slide over a light for 3 minutes. A smooth red suspension indicates satisfactory reagents.

(2) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. If whole blood is to be used, it must be used WITHOUT anticoagulant.

d. Procedure:

(1) Place 1 drop of UNDILUTED patient's serum (or 3 drops of whole blood WITHOUT anticoagulants) on a slide.

(2) Place 1 drop of positive control serum in another section of the slide.

(3) Add 1 drop of eosin reagent to each serum and mix each with a separate stirrer.

(4) Gently resuspend the latex reagent and add 2 drops to each serum-eosin mixture. Mix well and spread each mixture over an area about 1 inch square.

(5) Rock the slide gently for 3 minutes and examine over a light for agglutination of the latex particles.

e. Reporting Results. If the positive control serum gives the expected results (agglutination), report the results of the patients' sera as follows:

(1) No agglutination—Negative.

(2) Agglutination—Positive.

f. Sources of Error. In general, the same sources of error listed in paragraph 11-2f also apply to this test, with the following additions and exceptions:

(1) UNDILUTED serum must be used in this test. Whole blood without anticoagulants may also be used.

(2) The eosin reagent must be added to the serum before the latex suspension is added. False-positive results frequently occur if this order is reversed.

11–4. Latex Macroscopic Tube Test:

a. Principle. A suspension of globulin-coated latex particles is added to serially diluted serum in test tubes. After incubation at times and temperatures specified by the kit manufacturers, the reciprocal of the highest dilution of serum to cause agglutination of the latex particles is reported as the titer of rheumatoid factors in the serum.

b. Reagents and Equipment. Available commercially from several sources.

(1) Patient's serum, fresh.
(2) Latex-globulin reagent, as specified.
(3) Buffer diluent, as specified.
(4) Test tubes, 12 × 75 mm.
(5) Pipets, serological, 1 ml.
(6) Test tube rack.
(7) Incubator, 37° or 56°C, as specified.
(8) Refrigerator, 2°–10°C, if needed.
(9) Centrifuge, if needed.

c. Preliminary Steps:

(1) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. Frozen serum should be at room temperature for testing.

(2) If required, prepare the buffer diluent as specified by the manufacturer.

(3) If the latex particles require sensitization with globulin, prepare this reagent. NOTE: Some manufacturers suggest that the sensitization procedure be started on the day before the test is to be performed.

d. Procedure:

(1) Place 10 labeled test tubes in a rack.

(2) Pipet 1.9 ml of buffer diluent into the first tube and 1 ml into the remaining nine tubes.

(3) Pipet 0.1 ml of serum into the first tube, mix the contents, and transfer 1 ml of serum-buffer mixture to the second tube. NOTE: For greatest accuracy, this pipet may be discarded and a new one used for each succeeding tube.

(4) Mix the contents of the second tube and transfer 1 ml to the third tube, repeating this procedure through the ninth tube, from which 1 ml is discarded. The tenth tube is the control and receive no serum.

(5) Add 1 ml (or 1 drop, if specified) of latex-globulin reagent to each tube and shake the rack to mix the contents.

(6) Incubate the tubes as specified by the latex-globulin manufacturer.

(7) If specified, centrifuge the tubes for 3 minutes at 2,300 rpm.

(8) Gently agitate the tubes while observing for macroscopic agglutination of the latex particles. Record the highest dilution to show agglutination. This procedure is outlined in table 11-1.

e. Reporting Results. Report the reciprocal of the highest serum dilution to show agglutination as the titer. The titer does NOT include the dilution of the serum caused by adding the latex-globulin reagent.

f. Sources of Error. The sources of error listed in paragraph 11-2f apply to this test, but special attention must be paid to the specific manufacturer's instructions due to the many minor (but significant) variations available.

11–5. Sensitized Sheep Cell Slide Test:

a. Principle. Patient's serum is mixed with stabilized sheep erythrocytes that have been coated (sensitized) with gamma globulin. Agglutination of the erythrocytes indicates the presence of rheumatoid factors in the patient's serum.

Table 11–1. Latex Macroscopic Tube Test for Rheumatoid Factors.

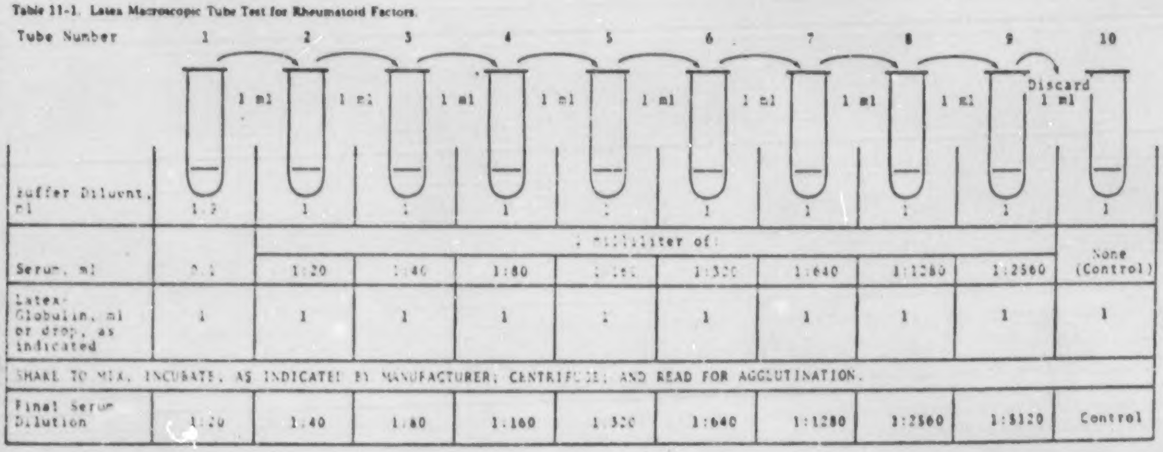


Table 11-1. Latex Macroscopic Tube Test for Rheumatoid Factors.

Tube Number	1	2	3	4	5	6	7	8	9	10
Buffer Diluent, ml	1.9	1	1	1	1	1	1	1	1	1
		1 milliliter of:								
Serum, ml	0.1	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	None (Control)
Latex-Globulin, ml or drop, as indicated	1	1	1	1	1	1	1	1	1	1
SHAKE TO MIX, INCUBATE, AS INDICATED BY MANUFACTURER; CENTRIFUGE; AND READ FOR AGGLUTINATION.										
Final Serum Dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control

b. Reagents and Equipment. Available commercially in kit form.

(1) Patient's serum, fresh or frozen.

(2) Positive and negative control sera.

(3) Sensitized sheep erythrocyte reagent.

(4) Saline, 0.9%.

(5) Glass or paper slides.

(6) Capillary tubes, calibrated, with bulb.

(7) Stirrers.

c. Preliminary Steps:

(1) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. Allow frozen sera to warm to room temperature and mix thoroughly before testing.

(2) Allow all reagents and control sera to warm to room temperature before testing.

(3) When a new lot of sensitized sheep erythrocyte reagent is opened, test the reagent with the positive and negative control sera according to the procedure below as a check on the suitability and proper storage of the reagent.

d. Procedure:

(1) With the calibrated capillary, transfer a measured amount of patient's serum (or control serum, during the reagent check) to a slide.

(2) Resuspend the sensitized sheep erythrocyte reagent and add 1 drop to each serum.

(3) Using a clean stirrer for each serum, mix each serum with the reagent and spread the mixture.

(4) If paper cards are used, rock the card for 30 seconds and let it stand undisturbed and examine for agglutination at the end of 2 minutes. With glass slides, rock the slide for 2 minutes and examine for agglutination.

(5) If no agglutination with the patient's serum occurs, the test is reported as—Negative.

(6) If agglutination occurs, prepare a 1:10 dilution of serum in 0.9% saline (1 drop of serum and 9 drops of saline) and repeat the test.

e. Reporting Results. If the control sera give the expected results, report the results as follows:

(1) No agglutination with undiluted serum—Negative.

(2) Agglutination with undiluted serum only—Positive, Undiluted Only. NOTE: This result may also be due to heterophile antibodies as well as low-titered rheumatoid factors, such as may be seen with a number of diseases other than rheumatoid arthritis.

(3) Agglutination with both undiluted and diluted serum—Positive.

f. Sources of Error. The sources of error listed in paragraph 11-2f also apply to this test, with the following additions and exceptions:

(1) Do not freeze the erythrocyte reagent because hemolysis may occur.

(2) Be sure to bring all reagents and sera to room temperature before testing.

(3) The possibility of the presence of heterophile antibodies must be considered in all positive tests.

(4) Undiluted, unheated serum at room temperature must be used for this test.

11-6. Interpretation of Results. These tests for rheumatoid factors should not be considered to be specific for the diagnosis of rheumatoid arthritis. In fact, only about 80 percent of the cases of classical rheumatoid arthritis produce detectable levels of rheumatoid factors, with the actual percentage varying from test to test. In addition, significant percentages of several other diseases produce these factors. However, these tests are still useful when their limitations are considered in interpreting results.

a. Sensitivity and Specificity of Tests. In general, the latex tests are more sensitive detectors of rheumatoid factors than the tests using erythrocytes. By contrast, the erythrocyte tests have fewer false-positive results so that positive erythrocyte tests more likely indicate rheumatoid arthritis than a positive latex test does.

(1) False-Negative Tests. About 20 percent of apparently classical cases of rheumatoid arthritis fail to produce detectable levels of rheumatoid factors when tested by the usual tests. Although the exact cause of false negatives is unknown, it has been found that these patients have a predominance of IgG antiglobulins rather than IgM as seen in positive patients. It has been suggested that these false negatives really are a different disease entity.

(2) False-Positive Tests. Most of the diseases that cause false-positive tests for rheumatoid factors are chronic diseases. As a rule, only a certain percentage of these cases will be positive, and usually only weakly positive. Some of these diseases are subacute bacterial endocarditis (SABE), syphilis, Hansen's disease, sarcoidosis, tuberculosis, cancer, and liver diseases, such as cirrhosis and viral hepatitis. In addition, most types of leukemia cause false positives, with some types about 100-percent positive. The false positives found in lupus erythematosus (LE) are interesting, because a significant number of cases of rheumatoid arthritis, in turn,

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cause false-positive serotests for LE. In general, false-positive tests for rheumatoid factors might be expected in any disease that results in a hypergammaglobulinemia.

b. **Interpretation of Titration Tests.** The minimum significant titer of rheumatoid factors varies from kit manufacturer to manufacturer, with some suggesting 1:20 (or 1:40) or greater on unheated serum as significant and 1:160 or greater on heated serum. Therefore, the manufacturer's instructions must be consulted in interpreting these tests. However, regardless of the test method used, the height of the titer of rheumatoid factors cannot be correlated directly with the severity of the disease, but a rise in titer helps confirm a diagnosis of rheumatoid arthritis. Titers generally persist for the life of the patient. Those diseases that cause false-positive screening tests for rheumatoid factors usually have low titers in titration tests. Frequently, high titers are found in apparently normal individuals who have an increased tendency for developing rheumatoid arthritis in the future. The use of titration tests may also be useful in identifying patients with hyperviscosity of the blood due to hypergammaglobulinemia.

CHAPTER 13

TESTS FOR PREGNANCY

13-1. Significance of Human Chorionic Gonadotropin (HCG):

a. Introduction. Human chorionic gonadotropin (HCG) is a hormone that is produced by the placenta during pregnancy and found in several body fluids, including blood and urine. It is also produced by certain rare tumors associated primarily with the reproductive organs of both males and females. With the exception of these rare tumors, pregnancy is the only condition to consistently produce HCG. The detection of HCG in urine or serum provides the basis for pregnancy tests.

b. Types of Tests for HCG. Until the past decade, pregnancy tests were based on the effects of HCG on the reproductive systems of various animals. Bioassay was necessary, because no other practical laboratory tests were available that were sensitive enough to detect the minute amounts of HCG found in the blood or urine of pregnant women. Over the years, rats, mice, rabbits, and a variety of frogs and toads were used with varying degrees of success. However, following the isolation and purification of HCG and the discovery that it was antigenic in rabbits, immunologic tests for pregnancy have essentially replaced the use of animals. Except for a radioimmunoassay test for HCG, nearly all of the immunologic tests for pregnancy presently in use are based on the inhibition of agglutination of either latex particles or erythrocytes that have been coated with HCG. A wide variety of these tests are available commercially in kits.

(1) In most of these tests, the patient's urine is, first, mixed with known HCG antiserum from rabbits. The next step is to add the HCG-coated latex particles or erythrocytes to the mixture. If HCG is present in the urine, the HCG antiserum will react with this urinary HCG, thereby inhibiting subsequent agglutination of the latex particles of erythrocytes. Conversely, agglutination indicates the absence of urinary HCG—or a negative pregnancy test.

(2) In addition, many of these tests can be performed on serially diluted serum or urine to provide quantitative HCG results in international units (I.U.) per 24 hours or per liter. The I.U. is based on the activity of an aliquot of a chorionic gonadotropin standard maintained by the World Health Organization.

c. Sensitivity of Pregnancy Tests. Aside from the obvious requirement to maintain an animal colony, the main disadvantage of animal tests for HCG is the variable sensitivity of these animals in detecting HCG. Sensitivity varies even between animals of the same species and also with the seasons of the year in some species. Immunologic HCG tests, on the other hand, are more readily standardized and more sensitive than animal tests. For example, radioimmunoassay can detect as little as 15 I.U. of HCG/liter, while the erythrocyte tests have a sensitivity of about 1,000 I.U./liter. Even the relatively insensitive latex tests, which have a sensitivity of 2,000 or more I.U./liter, can detect smaller amounts of HCG than can most animal tests. The actual sensitivity of each test kit is specified by the manufacturer, but a sensitivity of about 1,000 I.U./liter permits early diagnosis of pregnancy without being so sensitive that the small quantities of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in the urine cause a significant number of false positives.

d. HCG in Pregnancy. Pregnancy tests with a sensitivity of about 1,000 I.U./liter will first become positive about 4-7 days after the expected starting date of the first-missed menstrual period. These tests are nearly always positive within 3 weeks. In normal pregnancies, HCG titers on first morning urines rise rapidly and peak at about 100,000 I.U./liter in about 70 days after the last menstrual period. Then, titers fall and level off at about 25,000 for the duration of the pregnancy. Rapidly falling titers or titers of less than 5,000 usually precede abortions. Low titers of HCG are also frequently seen in ectopic pregnancies. With normal deliveries, these pregnancy tests usually revert to negative in about a week. Persistence of HCG after delivery may indicate a pathological condition.

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c. HCG in Other Conditions. High titers of HCG, that persist more than a hundred days after a missed menstrual period, may indicate the development of a hydatidiform mole or, more rarely, choriocarcinoma. Complete removal of a mole should result in pregnancy tests becoming negative in about a week. However, a persistently positive pregnancy test a week later may indicate either incomplete removal of the mole or an accompanying choriocarcinoma. In addition, teratomas of the testes and ovaries frequently cause positive pregnancy tests due to HCG production.

13-2. Latex Agglutination Inhibition Slide Tests. Many manufacturers produce pregnancy test kits based on latex agglutination inhibition. In selecting the kit to use, be sure to consider the sensitivity of the method in the decision. As a rule, latex pregnancy tests are less sensitive than hemagglutination tests in detecting HCG. Because of the number of manufacturers producing pregnancy test kits based on latex agglutination inhibition, the procedure and information in this paragraph will be presented in general terms only. For specific instructions, refer to the manufacturers' instructions.

a. Principle. Patient's urine is mixed on a slide with HCG antiserum, usually from rabbits. Then a drop (or two) of a suspension of HCG-coated latex particles is mixed with the urine-antiserum mixture, and the mixture is spread over an area prescribed by the manufacturer. The slide is rotated manually and examined within 2 minutes for agglutination. If HCG is present in the urine, the HCG antiserum is neutralized and agglutination of the HCG-coated latex particles does not occur—a positive pregnancy test. Conversely, if no HCG is present in the urine, the antiserum is not neutralized, and agglutination occurs—a negative pregnancy test.

b. Reagents and Equipment. Except for the items needed for urine collection, all items are usually supplied with the test kit:

(1) Patient's urine, fresh or frozen.

(2) Human chorionic gonadotropin antiserum (rabbit). Refrigerate, but do not freeze.

(3) HCG-coated latex suspension. Refrigerate, but do not freeze.

(4) Known positive and negative urines, collected locally and stored frozen in small aliquots.

(5) Pipets, disposable, size as specified.

(6) Glass slide, provided.

(7) Toothpicks or similar stirrers.

(8) Light source, preferably fluorescent.

c. Preliminary Steps:

(1) Collect the patient's urine. First morning specimens are preferred due to concentration of the HCG, but random samples are usually acceptable; if positive. Refrigerate or freeze speciments that cannot be tested in a few hours. Specimens should be at room temperature and well mixed for testing. Turbid urines may be centrifuged, and the supernate tested.

(2) Allow all reagents to warm to room temperature before testing.

(3) Test newly opened reagents with known positive and negative urines.

d. Procedures. Be sure to follow manufacturers' instructions if at variance with the following steps:

(1) Using the pipets provided with the kit, place 1 drop of urine on a section of the slide provided with the kit.

(2) Add 1 drop of HCG antiserum to the urine, mix thoroughly with a stirrer, and rotate the slide for 30 seconds.

(3) Using the dropper provided with the reagent, add 1 drop (or 2, if specified) of HCG-coated latex suspension to the urine-antiserum mixture. Mix thoroughly with a stirrer and spread the urine-antiserum-latex mixture over the entire designated area of the slide.

(4) Rotate the slide as specified and observe for agglutination of the latex particles under a direct fluorescent light at the end of 2 minutes.

e. Reporting Results:

(1) Agglutination within 2 minutes—Negative.

(2) No agglutination within 2 minutes—Positive.

f. Sources of Error:

(1) Freezing, as well as prolonged storage at room or higher temperatures, can destroy the reactivity of the reagents, so refrigerate them.

(2) Failure to allow reagents and urine to warm to room temperature before testing may result in false-positive (no agglutination) results.

(3) Blood or protein in the urine may inhibit agglutination of the latex particles, thereby causing false-positive results.

(4) Do not use reagents beyond the expiration date indicated by the manufacturer.

(5) Do not mix reagents from different manufacturers or even lot numbers of the same manufacturer.

(6) Care must be exercised in interpreting results of agglutination inhibition tests, because, unlike most serologic test results, a

positive result is indicated by no reaction and vice versa.

(7) Reagents must be thoroughly mixed for valid results.

(8) All glassware must be scrupulously clean and free of detergents for valid results.

(9) Drying of the reagents on the slide may simulate agglutination.

(10) Slides must be examined within the time specified by the manufacturer.

(11) Patient's serum must NOT be tested by this method. Use a hemagglutination inhibition method.

(12) First-morning urine specimens will yield more positives than random specimens due to concentration of the HCG.

g. Discussion:

(1) Known positive and negative urine specimens may be tested periodically as reagent controls. These urines may be dispensed in small aliquots and stored frozen. Once thawed, the aliquots should be discarded.

(2) Also available commercially is a kit based on latex agglutination inhibition with the antiserum and HCG-coated latex dried on cards. This kit requires water as the only reagent, in addition to urine. Another advantage of this test is that no refrigeration is required.

(3) Other latex tests for pregnancy, based on the direct agglutination of antiserum-coated latex particles, are available commercially. In these tests, urinary HCG agglutinates the latex particles; consequently, agglutination indicates pregnancy (the presence of HCG).

13-3. Hemagglutination Inhibition Tests. Because of the number of manufacturers producing pregnancy kits based on this principle, the procedure and information in this paragraph will be presented in general terms only. In selecting the kit to use, be sure to consider the sensitivity of the method in the decision.

a. Principle. Diluted patient's urine (or specially treated serum) is mixed with liquid or freeze-dried HCG antiserum in a test tube. Then HCG-coated erythrocytes are added, or freeze-dried erythrocytes may have been included with the freeze-dried antiserum. The erythrocytes are allowed to settle, undisturbed, for 2 hours. Inhibition of hemagglutination, as evidenced by the formation of a ring of settled cells, indicates the presence of HCG in the urine—a positive pregnancy test. Conversely, hemagglutination, as evidenced by the formation of a uniform mat of settled cells, indicates a negative test.

b. Reagents and Equipment:

(1) Patient's urine, fresh or frozen, or properly treated serum.

(2) HCG antiserum, liquid or freeze-dried in combination with HCG-coated erythrocytes.

(3) HCG-coated erythrocytes, suspension or freeze-dried in combination with the antiserum.

(4) Diluent, as specified.

(5) Test tubes, size as specified. Tests using freeze-dried reagents are performed in the reagent containers.

(6) Pipets, serological or as provided.

(7) Test tube rack, preferably with mirrow.

c. Preliminary Steps:

(1) Collect patient's urine according to paragraph 13-2c. If required, dilute the urine using the diluent and quantities specified by the manufacturer. If serum is to be tested, treat the serum as specified by the kit manufacturer.

(2) Test newly opened reagents with known positive and negative urines.

d. Procedure. The procedure outlined below is given in general terms only. Be sure to follow manufacturer's instructions:

(1) Add the specified volume of urine, diluted urine, or treated serum to the appropriate test tube.

(2) Add diluent, if required.

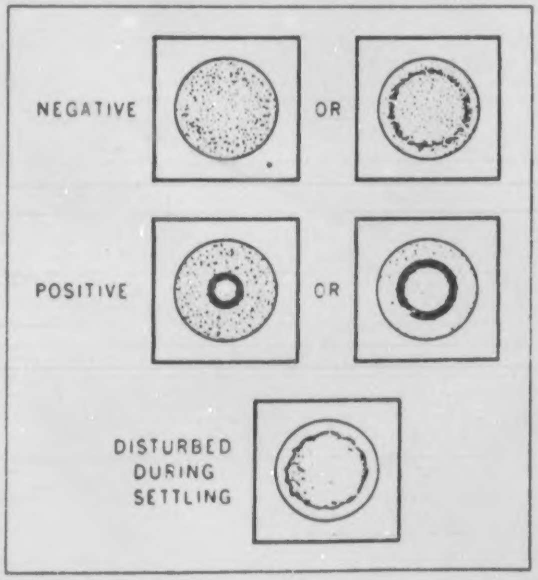


Figure 13-1. Reading Hemagglutination Inhibition Tests for HCG.

(3) With liquid reagents, add the specified amount of antiserum FOLLOWED by the specified amount of thoroughly resuspended, HCG-coated erythrocyte suspension.

(4) Gently mix the contents of the tubes by shaking and place the tubes in a rack. NOTE: Tubes must be exactly vertical.

(5) Let the tubes stand for 2 hours at room temperature in a place where they will be undisturbed by vibration and excessive heat.

(6) At the end of 2 hours, examine the tubes from the bottom (or with the aid of a mirrored rack) for agglutination as evidenced by the formation of a thin mat of settled erythrocytes covering the entire rounded bottom of the tube. Formation of a ring of settled erythrocytes indicates inhibition of hemagglutination. Tubes that have been disturbed during the settling period may show a ragged-edge layer of cells. Examples of possible reactions are depicted graphically in figure 13-1.

e. **Reporting Results:**

(1) Formation of a well-formed ring of cells (hemagglutination inhibition)—Positive.

(2) No ring (or a thin, jagged ring) of cells (no hemagglutination inhibition)—Negative.

f. **Sources of Error.** In general, the sources of error listed for the latex tests in paragraph 13-2f also apply to these tests. The following exceptions and additions should be noted:

(1) Use only test tubes specified by the manufacturer.

(2) The tubes must be exactly vertical and undisturbed during the 2-hour settling period.

(3) Be sure to use the diluent specified by the manufacturer.

(4) As a rule, urinary proteins do not interfere with these tests as much as with latex tests.

13-4. **Quantitative HCG Tests.** Titration of urinary HCG output is usually performed on 24-hour urine specimens with results reported in I.U. excreted/24 hours. As an alternative, first morning specimens may be tested and reported in I.U./liter of urine. Both latex and hemagglutination tests have been applied to quantitating HCG by serially diluting urine and testing each dilution as an individual specimen. The highest dilution to yield positive results is then used, along with the test sensitivity specified by the manufacturer and the volume of urine, to calculate the number of international units of HCG excreted/24 hours (or per liter on first morning specimens). For specific test procedures, follow the manufacturer's instructions.

13-5. **Interpretation of Results.** Most requests for pregnancy tests will require only a positive or negative result. Although the actual meaning of a positive or negative test varies with the sensitivity of the test procedure being used, most hemagglutination pregnancy tests will be positive when HCG concentrations exceed about one international unit/ml (1,000 I.U./liter) of urine or serum, but latex tests are much less sensitive. These same tests are negative at HCG concentrations below the sensitivity specified by the manufacturer.

a. **Normal Pregnancies.** Immunologic pregnancy tests usually become positive within the first week after the first-missed menstrual period and remain positive throughout the course of uncomplicated pregnancies. Occasionally, tests may revert to negative later in the pregnancy when HCG levels fall. After delivery, the tests usually become negative within a week. Persistance of a positive test more than a week after delivery most likely indicates a pathological condition, such as hydatidiform mole, choriocarcinoma, or gonadal teratomas.

b. **Ectopic Pregnancies.** Pregnancy tests are frequently negative during these pregnancies.

c. **Threatened Abortions.** Although pregnancy tests cannot be used as a reliable indicator of fetal death, falling HCG titers or titers of less than 3,000 I.U./24 hours (5,000/liter on first-morning urines) usually indicate inevitable abortion.

d. **Hydatidiform Moles.** These intrauterine tumors are usually associated with very high titers of HCG. These titers must be differentiated from the transient peak seen in normal pregnancies. Occasionally, these tumors are accompanied by low, but detectable, HCG titers. Following complete removal of the mole, pregnancy tests should revert to negative in about a week. In fact, a persistently positive test following attempted removal of the tumor may indicate either incomplete removal or, more seriously, choriocarcinoma.

e. **Choriocarcinoma.** These HCG-producing tumors may develop in conjunction with normal pregnancy, incomplete abortion, or hydatidiform mole. HCG titers are generally very high in these tumors, but these titers must also be differentiated from the transient peak seen in normal pregnancy.

f. **Ovarian and Testicular Teratomas.** These relatively rare tumors also produce HCG. Consequently, the usual pregnancy test may be requested—even on males.

g. False Negatives. Due to the high sensitivity (about 1 unit of HCG/ml) of immunologic pregnancy tests, false-negative tests are rare. However, if urine specimens are collected too early in the pregnancy, negative results may be obtained. In addition, threatened abortions and ectopic pregnancies are frequently associated with very low levels of HCG. Testing of first morning urines will help minimize the occurrence of false negatives.

h. False Positive. Positive pregnancy tests due to HCG produced by hydatidiform moles, choriocarcinoma, and related tumors should not be considered as false positive. However, occasional high urinary levels of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), may cause false positives. Although the sensitivity of immunologic pregnancy tests are adjusted to preclude most false positives, care must be exercised in interpreting pregnancy tests on urine collected during ovulation (high LH) and menopause (high FSH). In addition, blood or protein in the urine may cause false-positive results by inhibiting agglutination of the HCG-coated latex or erythrocyte suspension.

Chapter 15

TESTS FOR IMMUNOGLOBULINS AND COMPLEMENT

SECTION A—IMMUNOGLOBULINS

15–1. Significance of Immunoglobulins. Following electrophoresis of serum proteins, only the gamma globulin fraction is found to contain significant antibody activity. When the gamma globulin is injected into certain animals, the animals produce antibodies against the different antigens making up the gamma globulin. By this method, it has been found that gamma globulin is actually made up of five antigenically distinct globulins. These five globulins are referred to collectively as immunoglobulins, with the individual immunoglobulins designated as IgG, IgM, IgA, IgD, and IgE. For practical purposes these immunoglobulins may be considered to be synonymous with antibodies; therefore, alterations in the serum levels of immunoglobulins can have a bearing on the immunity and health of an individual. In fact, either an abnormally high or low serum level of a given immunoglobulin may indicate pathology. These immunoglobulin levels in the serum are estimated by using serologic methods, hence the inclusion of these methods in this manual.

a. **Production of Immunoglobulins.** All immunoglobulins are produced by lymphocytes in response to antigenic stimuli, such as pathogenic and saprophytic microorganisms, as well as other foreign substances that might gain entry into the body. In fact, except for the IgG received transplacentally from the mother, a newborn infant is essentially devoid of immunoglobulins unless it has contracted an intrauterine infection during pregnancy.

(1) The IgG level in the newborn is the same as that of its mother. Consequently, the infant has passively acquired immunity to essentially the same diseases as its mother; the infant is also susceptible to the same diseases. However, the IgG level of the infant falls to low levels in a few months, but during this time, the infant's immunologic system is maturing and commencing to produce all classes of immunoglobulins. By the middle teens, the IgG level is usually back up to the congenitally acquired level present at birth. In normal adults, this IgG comprises about 75 percent (by weight) of the total immunoglobulins.

(2) The other immunoglobulins—that is, IgM, IgA, IgD, and IgE—do not appear to cross the placental barrier in significant quantities. IgM may be produced before birth if the fetus should contract an intrauterine infection, but in general, all antibody (immunoglobulin) production starts after birth. Along with IgG, all of these immunoglobulins normally reach adult levels by the midteens. By that time, about 21 percent (by weight) of immunoglobulins will be IgA, seven percent—IgM, and less than one percent each of IgD and IgE.

b. **Characteristics of Immunoglobulins.** In addition to their antigenic differences, the five classes of immunoglobulins also vary considerably in their production and reactivity. For example, on the first stimulation by an antigen, most of the antibodies produced are IgM, but on a second contact with the antigen, mainly IgG is produced. Although both IgM and IgG may be produced in response to the same antigen, the reactivity of these two classes in serologic tests also varies significantly. IgG antibodies have been found to be especially effective as precipitating antibodies and complement fixing antibodies but less efficient than IgM in agglutination and hemolysis reactions. On the other hand, IgA, IgD, and IgE appear to have few implications in serologic reactions. The reactivity of the five classes also varies in the body. IgG, IgM, and IgA are the main classes that are active in immunity, while IgE appears to be most significant in allergic conditions. In addition to its presence in serum, IgA is also found in many body secretions, such as tears, urine, and bronchial secretions, where it most likely has an additional role in immunity. IgD is the only class of immunoglobulin whose function is essentially unknown.

c. **Immunoglobulin Deficiencies.** The body's inability to produce adequate quantities of one or all immunoglobulins may be inherited or acquired. Some infants may have hereditary agammaglobulinemia with production of all five classes severely depressed, or dysgammaglobulinemia with just one or two classes low and the others normal. Many of these infants become very susceptible to even the most common infections as soon as the maternal IgG levels have fallen. More frequently, a hypogammaglobulinemia is seen, where one or all classes of immunoglobulins may be moderately, but significantly, depressed. These individuals are also more susceptible to infections than normal. These low levels of immunoglobulins may be due to delayed maturation of the immunologic system of the infant or associated with other conditions. Severe malnutrition, nephrosis, and malignant lymphomas, such as lymphocytic leukemia and Hodgkin's disease, are just some of the conditions that can result in hypogammaglobulinemia and eventually affect the health of the individual. Identification of these individuals is important so that appropriate prophylactic measures can be practiced to prevent infection. A few diseases are characterized by below-normal levels of a single immunoglobulin.

d. Immunoglobulin Excesses. Several conditions may result in the overproduction of one or more of the immunoglobulins. For example, viral hepatitis usually shows excessive production of all immunoglobulin classes while another liver disease, Laennec's cirrhosis, shows only IgA and IgG increased. In contrast, hepatomas result in depressed levels of IgM. Systemic lupus erythematosus (SLE) is another disease associated with a general increase in serum immunoglobulins, but especially of IgG. In addition, several diseases are accompanied by significantly high levels of a single immunoglobulin. Examples are African sleeping sickness caused by *Trypanosoma gambiense* and a high IgM, hay fever and asthma—IgE, and multiple myeloma with very high levels of either IgG or IgA and rarely, IgM, IgD, or IgE. As can be seen from the diseases discussed above, abnormally high immunoglobulin levels may be just as significant as low levels.

e. Tests for Immunoglobulins. There are several immunologic tests for detecting the presence or absence of immunoglobulins, but the only practical laboratory method for estimating the concentration of the individual immunoglobulins is immunodiffusion. More specifically, single radial immunodiffusion is used. In this method, monospecific antibody produced in animals against one of the five classes of immunoglobulins is incorporated into the layer of agar gel. Then, the patient's serum (containing the immunoglobulin) is put in a well cut into the agar and the system is incubated, usually at room temperature, but the temperature depends on which test is being performed. The immunoglobulin diffuses into the antibody-containing agar and a ring of precipitate forms at the zone of optimum proportions of the patient's immunoglobulin (antigen here) and antibody. The diameter of the precipitate ring is proportional to the concentration of the particular immunoglobulin in the patient's serum. By simultaneously testing known standard reference sera, a quantitative result can be reported for the patient's immunoglobulin level.

(1) Several manufacturers make kits which include prepared agar plates, reference sera, and everything needed to perform these tests. Kits are available for all five classes of immunoglobulins as well as several other biologically significant proteins that may be found in serum and other body fluids in certain disease states. In addition, complement, to be discussed in the next section of this chapter, is also assayed by single radial immunodiffusion with all equipment available in kit form.

(2) Another very sensitive method for assaying small quantitites of these proteins is through the use of radioimmunoassay (RIA), but this method is beyond the capability of the average laboratory.

15-2. Immunodiffusion Tests for Immunoglobulins:

a. Principle. Patient's serum is put in a well cut into monospecific-antibody-containing agar gel in plates (usually commercially prepared). Simultaneously, three or four reference sera of known concentrations of the particular immunoglobulin under study are placed in other appropriately labeled wells. The plates are covered tightly and incubated according to the times and temperatures specified for the immunoglobulin. As the immunoglobulin diffuses into the antibody-containing agar, a ring of precipitate forms at the point of optimum proportions of antigen (immunoglobulin) and antibody. The diameter of the precipitate ring is proportional to the concentration of the specific immunoglobulin in the patient's serum. By plotting the diameter of the precipitate ring of each reference serum on the linear scale against its known immunoglobulin concentration on the logarithmic scale of two- or three-cycle semilogarithmic graph paper, the concentration of immunoglobulin in the patient's serum can be determined. NOTE: This reference curve MUST be prepared for each batch of tests to compensate for variations in incubation temperatures, humidity, agar gel lot variations, and other daily variables that might affect precipitate diameters.

b. Reagents and Equipment:

(1) Patient's serum.

(2) Reference sera, three or four different concentrations for each desired immunoglobulin.

(3) Immunodiffusion plates, various sizes with precut wells are available commercially.

(4) Moist chamber, such as a tightly covered jar or plastic bag containing wet gauze or filter paper.

(5) Capillary pipets or microliter syringes.

(6) Graph paper, two- or three-cycle semilogarithmic.

(7) Incubator, 37°C, needed for certain, but not all, immunodiffusion tests.

(8) Calibrated magnifying device for measuring ring diameters. Available commercially or, as an alternative, a dissecting microscope with an eyepiece reticle or stage micrometer may be used.

c. Preliminary Steps:

(1) Collect the patient's serum. Fresh serum is preferred, but frozen serum, that has not been repeatedly frozen and thawed, may be used.

(2) Remove immunodiffusion plates from the refrigerator. Plates containing visible moisture should be left open at room temperature for a few minutes before proceeding with the test.

d. Procedure:

(1) Identify wells for each patient's serum and reference serum. If more than one plate is required for the batch of tests, only one complete series of reference sera needs to be tested, but add one of the reference sera to each additional plate used in the batch.

(2) With a capillary pipet, CAREFULLY fill each well with the appropriate serum to exactly the top of the agar when viewed from the side. This is best accomplished by introducing the filled pipet to the bottom of the well and slowly withdrawing the pipet as the well fills. Do not overfill, underfill, introduce bubbles with the serum, or touch the sidewalls of the well with the pipet as these procedures may result in distortions in the precipitate rings or invalid results. NOTE: With practice, the capillary pipets may be filled to the exact point to accurately and rapidly fill the wells. If may be advantageous to mark this point, once it is determined, on new pipets. Since well sizes vary between kits, be sure to doublecheck filled wells.

(3) Cover the plates, put them into the moist chamber, and incubate as specified by the kit manufacturer. NOTE: If room temperatures fluctuate outside of the range of 20° to 25°C, use a constant temperature incubator.

(4) After incubation, measure and record the outside diameters (to the nearest 0.1 mm) of the precipitate rings. NOTE: Slightly oblong rings may be measured by averaging the diameters of the shortest axis and longest axis. Grossly irregular rings should be discarded, and the test repeated. Faint rings may be enhanced by dipping plates in 7.5 percent acetic acid for a minute or two and washing gently with cold water.

(5) Prepare a reference curve by plotting the ring diameter (or average diameter) of each reference serum on the linear (horizontal) scale against its immunoglobulin concentration on the logarithmic (vertical) scale of two- or three-cycle semilogarithmic graph paper. For ease in reading unknown results, use as much of the paper as possible. Be sure to start labeling the bottom line of each logarithmic cycle with 1, 10, 100, or 1000 mg% as appropriate, and NOT with "zero". See figure 15-1 for an example of a reference curve. DO NOT use this curve.

(6) Draw a straight line to best fit the three or four reference points.

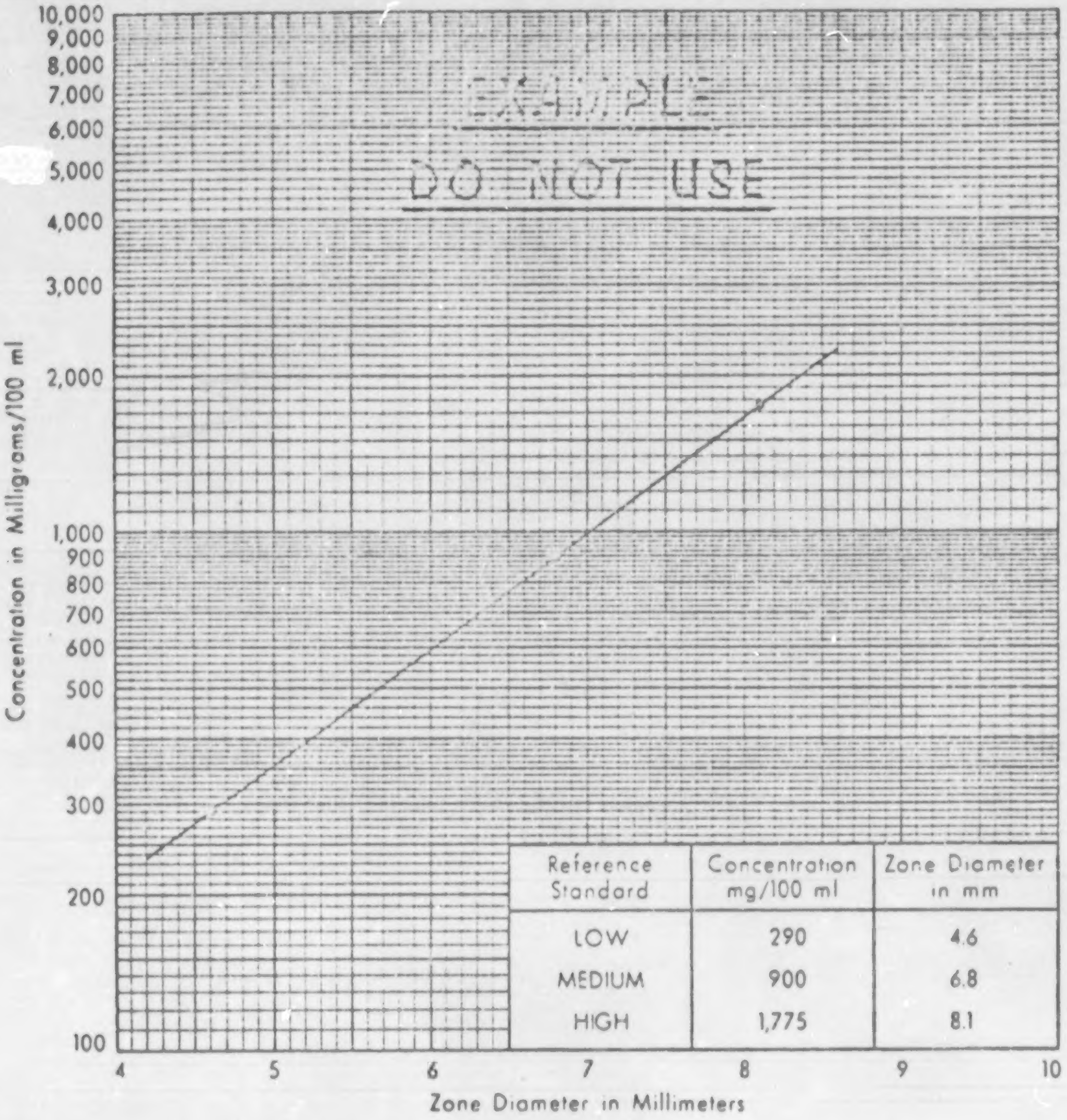
(7) Read the concentrations of the patient's sera from the reference curve. NOTE: If more than one plate is used and the value of the reference serum (one must be tested with each additional plate) does not fall on or very close to the reference curve, draw a line through the observed value parallel to the original curve to compensate for the variation in the individual plate. Then, read all patient's sera for that plate from the altered reference curve.

e. Reporting Results. Results may be reported in mg/100 ml of serum, or the recently proposed "Units of Activity" or International Units. In addition, the normal values for the test should be included on the report, because normals vary from kit manufacturer to manufacturer. In fact, ideally normal values should be developed by each laboratory, but one manufacturer's products and another's normal values should not be interchanged—they're probably significantly different.

f. Sources of Error:

(1) Use fresh serum for most reliable results. IgD appears to be especially altered by storage.

(2) Plates incubated longer than indicated by the manufacturer may yield invalid results. The diameters of the precipitate rings are proportional to the concentration



Reference Standard	Concentration mg/100 ml	Zone Diameter in mm
LOW	290	4.6
MEDIUM	900	6.8
HIGH	1,775	8.1

Figure 15–1. Example, Reference Curve for IgG.

of immunoglobulin at a given point in time, and the reactions are not allowed to go to completion.

(3) A complete reference curve must be prepared for each batch of tests to compensate for daily variations in test conditions. In addition, but sure to test one reference serum in each additional plate used in a batch to detect individual variations between plates.

(4) For most valid results, the values of unknown sera should fall within the range of concentrations of the reference sera. Several manufacturers make kits for the detection of low levels of immunoglobulins. Excessively high levels of immunoglobulins may be assayed by first diluting the serum with isotonic saline and then multipiying the observed resuits by the appropriate dilution factor.

(5) Be sure to use constant-temperature incubators if temperatures fluctuate.

(6) Wells must be filled as specified, or invalid results may result.

g. **Discussion.** With minor variations in incubation times and temperatures, single radial immunodiffusion tests are available commercially for all five immunoglobulins as well as the following proteins: albumin, transferrin, haptoglobin, ceruloplasmin, alpha-2-macroglobulin, complement C'3 (covered in the next section of this chapter) and a few others. In addition, some of the tests have been made more sensitive through the use of radioisotopes.

15-3. Interpretation of Results. Normal values of serum immunoglobulins vary over a wide range from individual to individual—even among apparently normal adults. More significantly, the age of the individual must be considered when interpreting results of these tests, because all immunoglobulins, except IgG, are essentially nonexistent at birth, but reach adult levels by the midteens or later. The exception, IgG, is at adult levels at birth, but then falls to low levels in a few months only to begin rising again to eventually reattain adult levels by the midteens.

a. Another important consideration in interpreting the results of these tests is that normal values vary, depending on which manufacturer's test kits were used. Ideally each laboratory should develop its own normal values for each age and immunoglobulin. However, if developing normal values is not feasible for a given laboratory, the normal values of the specific kit manufacturer must be followed. Do not mix normal values between manufacturers; they might vary significantly.

b. Depending on the immunoglobulin and the age of the patient being tested, either an abnormally high or low serum level might indicate a pathologic condition. Whether a result is normal or abnormal must be decided in light of the normal values used by the individual laboratory. Consequently, the conditions listed below for each immunoglobulin are considered to be associated with a relative increase or decrease, the individual results must be analyzed with reference to the normals established by the individual laboratory.

(1) Conditions With Generalized Alterations of Immunoglobulin Levels:

Increased	Decreased
Infectious hepatitis	Nephrosis
Lupus erythematosus	Malnutrition
Rheumatoid arthritis	Agammaglobulinemia, hereditary
	Hypogammaglobulinemia, transient or acquired
	Protein-losing enteropathy
	Malignant lymphomas

(2) Conditions With Altered IgG Levels:

Increased	Decreased
IgG-myeloma	Mongolism
Hyperimmunization	
Microbial infections	
Laennec's cirrhosis	

(3) Conditions With Altered IgM Levels:

Increased	Decreased
IgM-myeloma (rare)	Hepatomas
Trypanosomiasis	
Congenital infections (at birth)	
Waldenstrom's macroglobulinemia	

(4) Conditions With Altered IgA Levels:

Increased	Decreased
IgA-myeloma	Some normal individuals
Laennec's cirrhosis	Ataxia telangiectasia
	Gastrointestinal disease

(5) Conditions With Altered IgD Levels:

Increased	Decreased
IgD-myeloma (rare)	Importance unknown

(6) Conditions With Altered IgE Levels:

Increased	Decreased
IgE-myeloma (rare)	Importance unknown
Hay fever	
Allergic asthma	
Eczema	
Ascaris infestation	

SECTION B—COMPLEMENT

15-4. Significance of Complement. Complement (C') is a collective term for a group of eleven serum proteins that react sequentially in the presence of certain antigen-antibody complexes. Some of the components of C' are heat labile; thus heat inactivation of serum is a simple means of destroying complement activity for serologic tests where C' might interfere with results. In reactions with some antigen-antibody complexes, all eleven C' components may be involved; in others, only a few of the eleven are needed. Several types of antigen-antibody complexes can deplete one or more of the C' components from serum. In effect, antigen-antibody reactions inactivate complement as readily as heat does. This depletion of complement by antigen-antibody complexes is the basis for the complement fixation test described briefly in chapter 2. In addition to complement fixation, the activities of C' and its components have been studied in relation to several in vitro and in vivo situations. Several diseases are known that show alterations in either the activity or serum level of C' or its components. The detection of these variations is primarily by single radial immunodiffusion.

a. Lytic Activities of Complement. One of the most widely studied functions of C′ is its role in the lysis of certain cells that have been reacted with specific antibody. For example, many gram-negative bacteria may be agglutinated by antibody alone, but complement, along with antibody, amy cause bacteriolysis. Antibodies and C′ are also involved in the in vitro lysis of several types of tumor cells and erythrocytes. In fact, hemolysis of antibody-coated erythrocytes has been used as an assay method for total C′ activity. The correlation of these in vitro C′ activities with similar in vivo activities is difficult, especially with regard to the role of C′ in immunity. However, C′ does seem to be involved in certain acquired hemolytic anemias, such as paroxysmal cold hemoglobinuria. In these cases, C′ activity in the serum drops significantly.

b. Nonlytic Activities of Complement. In addition to its lytic activities, C′ also takes part in several other antigen-antibody reactions. Some of these reactions would appear to have a significant effect on the immunity of an individual. For example, C′ has been found to be active in opsonization, immune adherence, and chemotaxis, all of which help promote the phagocytosis of invading microorganisms and other foreign particles. Increased permeability of blood vessels is another C′ associated observation that may enhance an individual's ability to combat disease by allowing antibodies and other antimicrobial factors to diffuse more readily to the invasion site.

c. Diseases With Altered Complement Levels. Total C′ activity has been studied in a wide variety of diseases, but very few of these diseases have abnormal C′ levels consistently enough to be useful diagnostically. Several infectious diseases have been found to have low total C activities late in the disease, but there are other more specific and practical means for diagnosing these infections. Although kits are available commercially for assaying total C′ activity, these tests have not gained wide acceptance. The problem is that a low total C′ level does not identify the abnormal component (or components), and frequently an individual component may be significantly abnormal while total C′ activity appears normal. In addition, the test must be performed immediately after the serum is collected. By contrast, the individual C′ component levels have been studied in only a few diseases, and C′3 is the only component for which the practical laboratory test is commercially available. C′3 is assayed by single radial immunodiffusion—the same test used for immunoglobulins in the previous section of this chapter. Although deficiencies in other C′ components have been related to certain diseases, only C′ deficiencies are covered in this manual.

(1) Acute Poststreptococcal Glomerulonephritis. Low serum levels of C′3 have been found in this disease. The low levels are most likely due to the immunologic reaction of antibodies and C′3 with tissues of the kidneys, especially the glomeruli. Both gamma globulin and C′3 have been found in glomerular tissue in this disease.

(2) Systemic Lupus Erythematosus (SLE). The low serum levels of C′3 in SLE is most likely due to the similar immunologic factors as discussed with acute poststreptococcal glomerulonephritis. The depression of C′3 levels becomes especially evident in SLE with renal involvement. C′3 levels may return to normal as the renal lesions heal. Sequential testing provides a method for following the severity of renal involvement in both SLE and acute poststreptococcal glomerulonephritis.

(3) Bacteremia by Gram-Negative Bacilli. A lower than normal level of C′3 is noted in severe cases of bacteremia caused by certain gram-negative bacilli. The depression of C′3 is especially correlated with a poor prognosis, such as a patient in shock or about to die.

(4) Chronic Liver Diseases. Diseases, such as chronic viral hepatitis and alcoholic cirrhosis, may have low C′3 levels, but levels return to normal with resolution of the disease.

(5) Graft Rejections. Low C′3 levels have been observed in severe cases of organ-transplant rejections. A progressively decreasing C′3 suggests a poor prognosis for retention of the graft.

15-5. Immunodiffusion Test for Complement C′3. This test is the same as the one given for immunoglobulins in paragraph 15-2, except that the agar gel contains antibody against complement C′3. Special emphasis should be made to use fresh serum for this test. Ideally, serum should be separated from the clot and tested immediately, but frozen serum may be used. All sources of error and other facets of the test have been covered in paragraph 15-2.

15-6. Interpretation of Results. Each laboratory should develop its own range of normal values for C′3, or as an alternative, should be sure to use the values specified by the kit manufacturer. C'3 test results are more useful when the test is performed serially as the disease progresses. See paragraph 15-4c for diseases with altered C'3 levels.

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SECTION IV - ANNEXES

ANNEX H

BASIC PROCEDURES IN PARASITOLOGY

OBJECTIVE: Given appropriate specimens, correctly use the most applicable concentration methods; apply the Giemsa staining principles in preparing blood smears, and microscopically identify selected parasitic organisms within an acceptable degree of accuracy.

Subject and LP No	Lesson Objectives	References
Introduction to Parasitology 08-280-601	Briefly discuss the course format and grading system. Define terms frequently used in parasitology.	Belding, p. 17; Cheng, pp 86, 120-125.
Parasitological Laboratory Techniques 08-280-602	List the types of specimens submitted for parasitology examinations. List the stain reactions for Iodine, MIF, Trichrone, and Iron hematoxylin. Descrive the handling and preserving of parasitology specimens.	TM 8-227-2, pp 1-1 to 2-3, 5-1 to 5-4, 7-1 to 7-4.
Protozoan Organisms: Class Rhizopoda and Zoomastigophora 08-280-603	Select facts about the characteristics of the classes Rhizopoda and Zoomastigophora. Select the specimen of choice, mode of infection, and infective form of each of the protozoans studied. After practice find and correctly identify the protozoans studied. After practice, differentiate the protozoans studied from pseudoparasites. List the identifying characteristics of each of the protozoans studied to include the cystic and trophozoite stages.	Belding, pp 31-299, 1218-1219; Brown, pp 17-68; Cheng, 161-233.
Formalin-Ether Concentration Technique 08-280-604	List the steps in performing the Formalin-Ether concentration technique.	TM 8-227-2, pp 5-7; Belding, pp 1170-1174; VT 61.

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Subject and LP No	Lesson Objective	References
The Class Cestoda 08-280-611	Select characteristics of the members of the class Cestoda. Select facts about the life cycle to include habitat, specimen, diagnostic forms, infective forms, intermediate hosts and mode of infection of the organisms studied. List identifying criteria for each of the organisms studied. After practice, find and correctly identify the Cestodes studied.	Belding, pp 564-644; Brown, pp 169-205; Cheng, pp 474-541.
The Class Trematoda 08-280-612	Select characteristics of the members of the class Trematoda. Select facts about the life cycle to include habitat, specimen, diagnostic forms, infective forms, intermediate host and mode of infection of the organisms studied. List identifying criteria for each of the organisms studied. After practice, find and correctly identify the Trematodes studied.	Belding, pp 645-795; Brown, pp 206-250; Cheng, pp 377-457.
Giemsa Stain Practical Exercise 08-280-613	Correctly prepare and stain a thick and thin blood film utilizing the Giemsa stain technique.	TM 8-227-2, pp 11-3 to 11-5; Belding, pp 1134-1142.
The Class Nematoda 08-280-614	Select characteristics of the members of the class Nematoda. Select facts about the life cyle to include habitat, specimen, diagnostic forms, infective forms, intermediate host and mode of infection of the organisms studied. After practice, find and correctly identify the nematodes studied.	Belding, pp 346-558; Brown, pp 99-168; Cheng, pp 586-689.

Subject and LP No		Lesson Objective	References
The Stool Specimen 08-280-605		Briefly discuss the test procedures performed on stool specimens. List and discuss the terms used to report results on the Standard Form 552.	TM 8-227-2, pp 1-4, 2-1 to 4-5; Belding, pp 1170-1174.
Identification of Organisms-Studied in all Classes 08-280-606		Given a simulated specimen with a diagnostic form of a parasitic organism studied, apply identification criteria to identify that organism. Given a prepared, mounted speciment containing diagnostic forms of parasites studied, find and identify the organisms present.	All previous references.
Malaria Organisms: Class Telosporea 08-280-607		Select characteristics of the class Telosporea. Select facts about the life cycle of the genus Plasmodium. List identifying criteria for the diagnostic forms of the genus Plasmodium. After practice, properly report whether a given stained blood smear is positive or negative for Plasmodium species.	Belding, pp 230-320; Brown, pp 43-44, 68-98; Cheng, pp 247-283.
Giemsa Stain 08-280-608		Select the steps in preparing and staining a thick and thin blood film using the Giemsa stain.	TM 8-227-2, pp 11-1 to 11-5.
Practical Examination #1 08-280-609		Correctly identify representative examples of selected organisms.	All previous references.
Introduction to the Helminths 08-280-610		Select the proper classification of each of the helminths studied. Define terms used in the description of the helminths. Compare the life cycles of the separate classes found in the helminths.	TM 8-227-2, pp 9-13 to 9-14; Belding, pp 3-11, 346-365, 564-571, 645-663.

Subject and LP No	Lesson Objective	References
Formalin-Ether Concentration Practical Exercise 08-280-615		
Practical Examination #2 08-280-616	Correctly identify representative examples of selected organisms.	All previous references.
Other Parasitic Organisms of Medical Importance 08-280-617	List other parasitic organisms among the Protozoa and Helminths that may be of possible medical importance. Select facts about the habitat and mode of infection of each of the parasitic organisms studied. Select methods of recovery for identification to include specimen of choice, and techniques for concentration and staining.	All previous references.
Final Written Examination 08-280-618	Answer questions concerning all material presented during the parasitology course.	All previous references.
Final Scan 08-280-619	Given stained blood smears, microscopically locate and determine the presence of the genus Plasmodium to report whether the smear is positive or negative for malaria with an accuracy of 70%. Microscopically locate and identify from prepared slides, members of all classes studied with at least 70% accuracy.	All previous references.

SECTION IV - ANNEX H (Cont)

BIBLIOGRAPHY

Belding, D. L. Textbook of Parasitology. 3d ed. New York: Appleton Century-Crofts, Inc., 1959.

Brown, H. W. Basic Clinical Parasitology. 3d ed. New York: Appleton Century-Crofts, Inc., 1969.

Cheng, T. C. General Parasitology. New York: Academic Press Inc., 1973.

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SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-601
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Introduction to Parasitology (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

TM 8-227-2, Aug 1974

Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp 17.

Cheng, T.C. General Parasitology. New York: Academic Press Inc., 1973, p 86, 120-125

STUDY ASSIGNMENT: After calss study GR 08-280-601-1

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601-1 issued to students at the beginning of the course

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: For centuries man has been engaged in a struggle to overcome his troublesome relationship with parasitic organisms. Some parasites are able to endure a wide variety of environmental extremes in order to survive. Since the tropics and subtropics offer more favorable conditions for their existence, parasitic infections have a higher incidence among populations in these areas. Primitive sanitary practices aid immensely in the spreading of parasitic diseases. Therefore, Armies in the field or troops stationed in endemic areas are constantly exposed to parasitic infections which may result in

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*This lesson plan supersedes LP 08-280-601, 017

a loss of many man-hours and, occasionally, in death. Correct identification of parasites by the laboratory specialist is necessary before control measures can be initiated.

B. Objectives:

1. Given a list of definitions of parasitological terms, select the definition which best corresponds to the terms as presented in class.

2. Given a list of protozoan cell components and unlabeled diagrams of various parasitic organisms, correctly match the cell components with the correct unlabeled items on each diagram.

3. Given characteristics of protozoans in the cystic and trophozoite stages, select the characteristics which correspond to the given morphological stage.

4. Given the basic classification schema of the parasitic organisms of medical significance, be prepared to list the taxonomic order of the common parasitic organisms as presented in class.

C. Class Procedure and Lesson Tie-in

This is an orientation to parasitology. It introduces the course, vocabulary in parasitology, classification of organisms and protozoan cell components.

II. EXPLANATION (30 min)

A. Overview of Parasitology Course

1. Course format

a. Lectures

b. Practical exercises

c. Quizzes and examinations

2. Course objectives

a. Crucial (Pass/Fail)

(1) Crucial written exams 1 and 2 - 90% accuracy

(2) Final scan malaria(4 slides) - 75% accuracy

(3) Final scan intestinal protozoans and helminth eggs and larva - 70% accuracy

/

(4) Crucial exams = 70 course points

b. Ranking

(1) Quiz average = maximum 5 course points

(2) Scope to scope = maximum 5 course points

(3) Final scan protozoans and helminths = maximum 10 course points

(4) Final written exam = maximum 10 course points

(5) Total ranking points = 30 course points

c. Passing of course = passing all crucial material

B. Terms and Definitions Frequently Used in Parasitology

NOTE: Refer students to GR 08-280-601 (Glossary)

1. Parasitology - The study of parasites and their relationship to their host.

2. Parasite- An organism that lives in or on another organism called the host. It derives benefit from its association with the host but contributes nothing useful in return. Parasites may be pathogens or nonpathogens.

3. Host - Any animal(or plant)upon, or within, which another organism lives as a parasite.

   a. Definitive - The host in which a parasite undergoes its sexual or adult life stage.

   b. Intermediate - The host in which a parasite undergoes its asexual or larval stages.

   c. Reservoir - Clinically, the animal which replaces man in the normal life cycle of a parasite.

   d. Vector - A carrier(usually an arthropod)which transfers an infective agent from one host to another.

4. Autoinfective - A host is his own direct source of reinfection.

5. Infective form - The stage in the life cycle of a parasite which infects the host.

QUESTION: What is parasitology?

ANSWER: Parasitology is the study of parasites and their relationship to their hosts.

QUESTION: Man is, most likely, what kind of host?

ANSWER: Man is most likely to be the definitive host.

C. Classification

NOTE: A transparency showing the classification chart is shown on the overhead projector. Students may follow the chart in GR 08-280-601-1. Briefly discuss the classification schema.

D. Protozoan Morphological Stages

1. Trophozoite
   a. Motile
   b. Feeding
   c. Reproduces by binary fission
2. Cyst
   a. Non-motile
   b. Non-feeding
   c. Usually the infective form
   d. Reproduces by nuclear division

E. Protozoan Cell Components

NOTE: Refer to drawing of Protozoan Cell in GR 08-280-601

1. Cytoplasm
   a. Ectoplasm - cell locomotion organelles
      (1) Pseudopod
      (2) Flagellum
      (3) Cilia

b. Endoplasm - cell metabolism

2. Nucleus

a. Nuclear membrane

b. Karyosome

c. Chromatin granules

3. Inclusions

a. Glycogen vacuole - food storage

b. Chromatoidal bars - thought to be food storage

c. Bacteria

d. RBC's

F. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Overview of parasitology course

2. Terms and definitions frequently used in parasitology

3. Classification

4. Protozoan morphological stages

5. Protozoan cell components

B. Closing Statement: Parasitism is widespread. Each year millions of people suffer the ill effects of diseases caused by parasites. The discovery, prevention and treatment of parasitic infections forms a continuous chain in which the medical laboratory specialist provides an important link.

INSTRUCTIONAL HOURS

Number of instructors required --- 1

Primary Hours	1
Secondary Hours	0
TOTAL	1

175

GR 08-280-601-1
068

DEPARTMENT OF THE ARMY

ACADEMY OF HEALTH SCIENCES, U.S. ARMY

FORT SAM HOUSTON, TEXAS 78234

BASIC MEDICAL PARASITOLOGY

MEDICAL LABORATORY SPECIALIST (BASIC) COURSE

92B10

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Microbiology Branch
Laboratory Sciences Division

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SECTION A: GENERAL INFORMATION

I. Glossary of Parasitological Terms

1.	Abopercular:	Refers to the position opposite the operculum.
2.	Autoinfective:	Process of reinfection in which the patient is his own best source of infection.
3.	Axostyle:	The central support structure of a flagellate.
4.	Cercaria:	The final free-swimming larval stage of a trematode parasite, consisting of a body and a tail.
5.	Cestoidea:	The name of a class of metazoan organisms consisting of the tapeworms.
6.	Charcot-Leyden crystals:	Crystals which are greatly flattened in the longitudinal axis and terminate in needle-like points at their opposite poles. They are indicative of intestinal bleeding.
7.	Chromatin:	The dark staining portion of a cell nucleus which displays a characteristic pattern useful in species identification.
8.	Chromatoidal bar:	A dark staining bar found in certain cysts of the protozoans (amoebae).
9.	Commensalism	A form of symbiosis between members of two species in which one is benefited and the other is neither benefited nor harmed by the relationship.
10.	Cyclophyllidea:	An order of the tapeworms characterized by four sucking discs on the scolex.
11.	Cyst:	A protective form of certain protozoan organisms and a means of transmission of these organisms.
12.	Cysticercosis:	The condition of being infected with cysticercus larvae.
13.	Cysticercus larva:	A larval form of tapeworm, consisting of a single scolex enclosed in a bladder-like cyst.
14.	Cytoplasm:	The protoplasm of the cell other than that of the cell nucleus.
15.	Definitive host:	The host which harbors the adult or sexually reproducing form of the parasite.

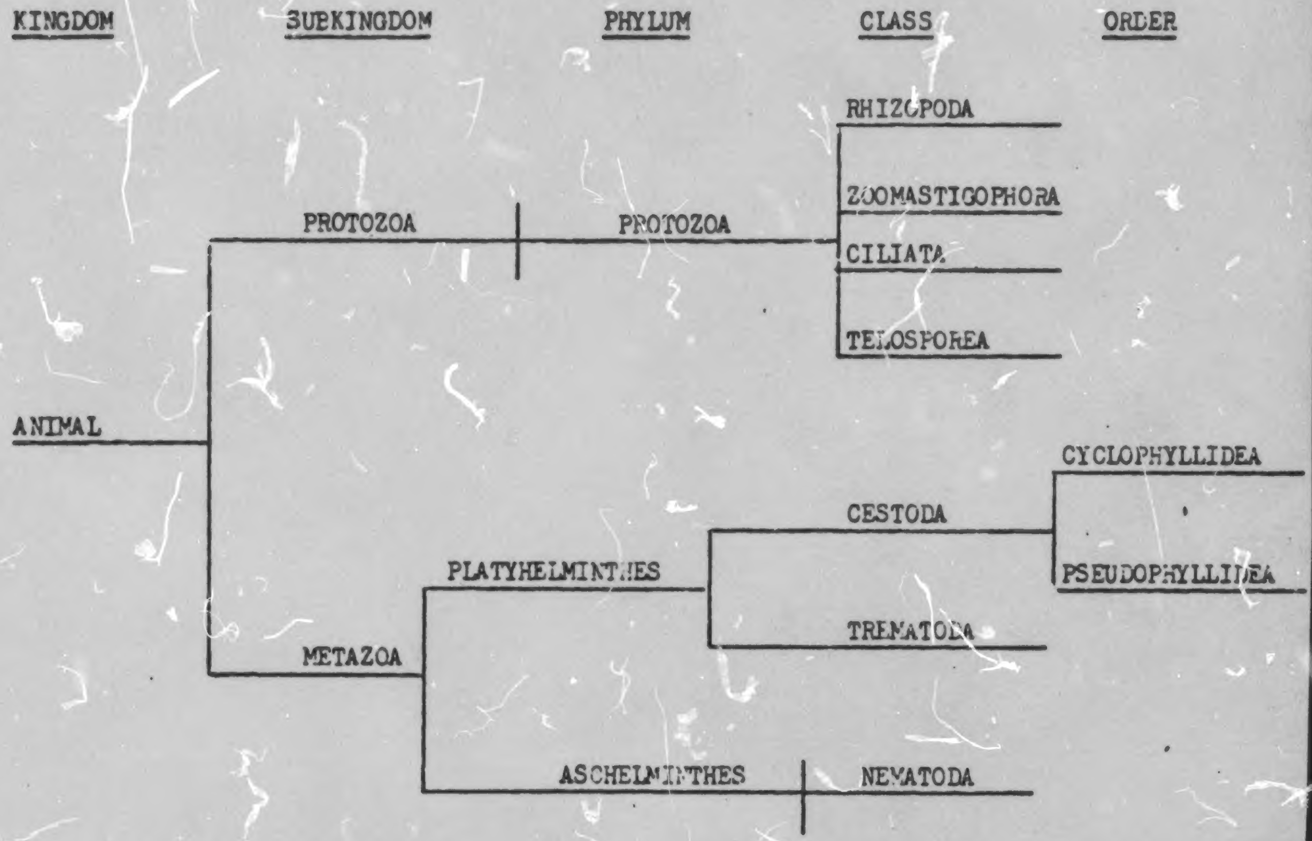
16. Diecious:	Having male and female sex organs in separate individuals.
17. Ectoplasm:	The outer clear zone of cytoplasm on the immediate margin of the cell.
18. Embryophore:	A gelatinous layer surrounding the embryo.
19. Endemic disease:	A disease of low incidence that is always present in a community or country.
20. Endoplasm:	The inner granular zone of cytoplasm which makes up the greater mass of the cell.
21. Epidemic:	A disease of high incidence which attacks many people in a region at the same time.
22. Filariform: larva:	Threadlike larva; infective form of *Hookworm* species and *Strongyloides stercoralis*.
23. Flagellum:	A mobile, whiplike process, filamentous appendage originating in the cell wall or outer layers of cytoplasm, and serving as an organ of locomotion.
24. Fluke:	A common name used when discussing the organisms of the class Trematoda.
25. Gametocyte:	The sexual Plasmodial cell in malarial blood which may produce gametes when taken into the mosquito host. It may be male (microgametocyte) or female (macrogametocyte).
26. Geographic distribution:	That area or region of the world where an organism exists.
27. Genital primordium:	The earliest indications of the development of the sex organs.
28. Glycogen (mass):	Refers to a food storage vacuole found in certain cysts of protozoans. Usually observable when stained.
29. Gravid:	Pregnant or full of eggs.
30. Habitat:	The natural site in the host where a parasite lives.
31. Helminth:	A general term applicable to the various species of worms which may be parasitic to man.

32. Hemozoin:	The pigment formed by metabolic waste products of malarial parasites within the red blood cells.
33. Hexacanth oncosphere:	The six-hooked embryo of certain species of tapeworms which is liberated from the egg at the time it hatches.
34. Host:	An organism which harbors another organism (parasite).
35. Hyaline:	Glassy and transparent, or approximating this state.
36. Infective form:	Stage of the life cycle of a parasite during which it is capable of infecting the host.
37. Intermediate host:	The host which harbors the larval or the asexually reproducing form of the parasite.
38. Karyosome:	One of the spherical masses of chromatin in the nucleus of a cell, gererally situated at or near the center of the nucleus and more deeply staining with hematoxylin than the remaining nuclear chromatin.
39. Larva:	An immature stage in the life history of an animal in which it is unlike the parent.
40. Merozoite:	A young form of a malarial parasite derived after the splitting of a mature schizont.
41. Metacercaria:	The encysted resting or maturing stage of a trematode parasite in the tissues of an intermediate host.
42. Metazoa:	A multi-cellular organism.
43. Miracidium:	The free-swimming larva which emerges from the egg of a fluke at the time the egg hatches, and which penetrates the body of the snail host.
44. Mode of infection:	The means by which a parasite enters the host.
45. Monecious:	Having male and female sex organs in the same individual. This does not imply self fertilization.
46. Neck:	Area below the scolex which possesses germinative tissue from which the proglottids arise.

47. Nematoda:	The name of a class of metazoan organisms belonging to the phylum Ashelminthes and consisting of the true roundworms.
48. Nuclear membrane:	The thin outer layer which encloses the nucleoplasm of the nucleus.
49. Nucleus:	A spheroid body within a cell characterized by its dense structure, functioning in a "control" capacity in reproduction and other processes.
50. Operculum:	The cap which covers the opening in the shell of eggs of certain species of flukes and tapeworms.
51. Ova:	A general term for helminth eggs.
52. Parasite:	An organism which lives on or in another organism called the host. It benefits from the host, but contributes nothing beneficial to the host.
53. Parasitism:	An obligatory relationship in which one organism (the parasite) is metabolically dependent on another organism (the host). The host may or may not be harmed by the relationship.
54. Plerocercoid larva:	The worm-like completed larval stage of Pseudophyllidean tapeworms; found in the tissues of vertebrates and invertebrates.
55. Proglottid:	One complete segment (reproductive unit) of the strobila of a tapeworm.
56. Protozoan:	A single-celled (unicellular) organism which is composed of one or more nuclei surrounded by cytoplasm and is contained within a limiting cell membrane.
57. Pseudophyllidea:	An order of the tapeworms (class Cestoidea) characterized by an almond shaped scolex with a pair of sucking grooves (bothria).
58. Pseudopod:	A temporary protrusion of the ectoplasm of an amoeba; used for locomotion and for injestion of food.
59. Reservoir host:	An animal which harbors the infective form (to man) of a parasite.

60. Rhabditiform larva:	A minute, rod-shaped larval form of a worm belonging to the class Nematoda, usually living in damp earth, but occasionally found in man.
61. Rostellum:	The raised portion at the top of the scolex of certain tapeworms. May or may not be retractable.
62. Roundworm:	The common name for organisms belonging to the class Nematoda.
63. Schizogony:	The asexual cycle of the class Telosporea; particularly the asexual cycle of the malaria parasite in the blood corpuscles and liver cells of man.
64. Schizont:	A stage in the asexual cycle of the malarial parasite during which the chromatin divides several times before cytoplasmic division into individual merozoites occurs.
65. Scolex:	The "head" area where the organs of attachment of a tapeworm are located.
66. Sporogony:	The sexual cycle of the Telosporea; particularly the sexual cycle of the malarial parasite in the mosquito.
67. Sporozoite:	The infective form to man of the malarial parasite.
68. Strobila:	That portion of the body of a tapeworm which is composed of chains of proglottids.
69. Symbiosis:	The close association or living together of two organisms of different species for their mutual or one-sided benefit.
70. Tapeworm:	The common name for organisms belonging to the class Cestoda.
71. Trematoda:	The class of metazoan organisms consisting of the flukes.
72. Trophozoite:	The active, vegetative, feeding, motile stage of a protozoan.
Malarial trophozoite:	The stage of schizogony between the merozoite stage and the schizont stage.
73. Vacuole:	Any of the various small spaces or cavities which float about in the cytoplasm of protozoan cells. May contain glycogen, injested food, or other material.

74. Vector:	A carrier, usually an arthropod, which transfers an infective agent from one host to another.
75. Viable:	In a living state.


KINGDOM
SUBKINGDOM
PHYLUM
CLASS
ORDER
RHIZOPODA
ZOOMASTIGOPHORA
PROTOZOA
PROTOZOA
CILIATA
TELOSPOREA
ANIMAL
CYCLOPHYLLIDEA
CESTODA
PLATYHELMINTHES
PSEUDOPHYLLIDEA
TREMATODA
METAZOA
ASCHELMINTHES
NEMATODA

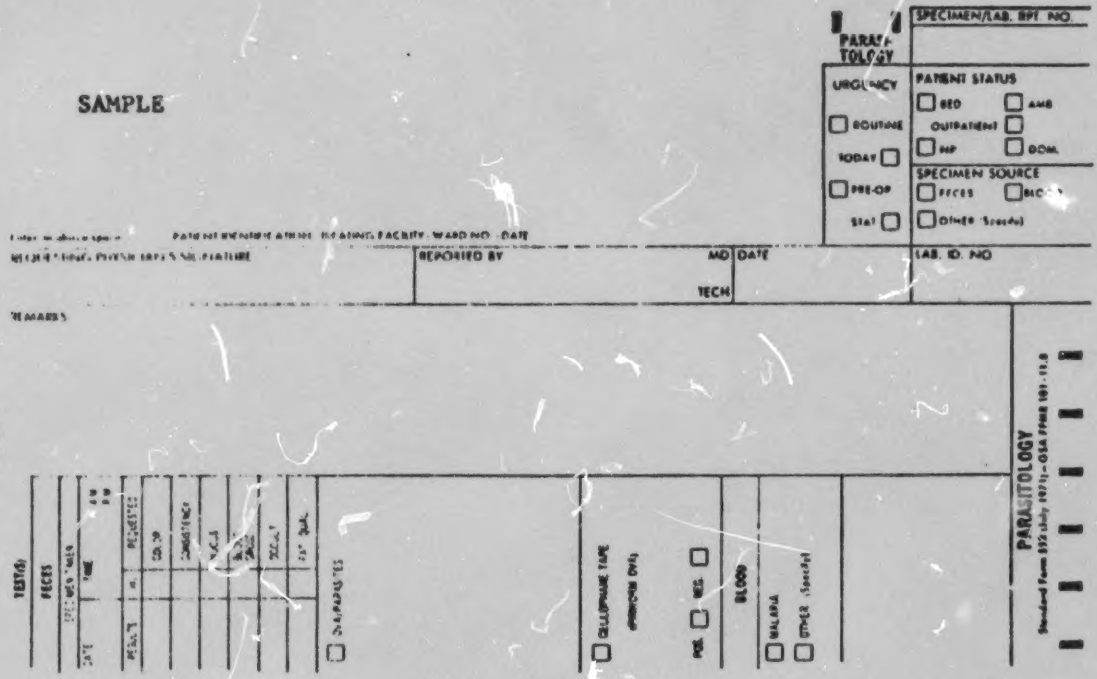
II. Classification Chart

SECTION B: LABORATORY TECHNIQUES

I. Macroscopic and Microscopic Examination of Feces

STANDARD FORM 552

SAMPLE



PARASITOLOGY

SPECIMEN/LAB. RPT. NO.

URGENCY

☐ ROUTINE

TODAY ☐

☐ PRE-OP

STAT ☐

PATIENT STATUS

☐ BED ☐ AMB

OUTPATIENT ☐

☐ NP ☐ DOM.

SPECIMEN SOURCE

☐ FECES ☐ BLOOD

☐ OTHER (Specify)

PATIENT IDENTIFICATION · TREATING FACILITY · WARD NO. · DATE

REQUESTING PHYSICIAN'S SIGNATURE

REPORTED BY

MD

TECH

DATE

LAB. ID. NO

REMARKS

TEST(S)

FECES

COLOR

CONSISTENCY

☐

CELLOPHANE TAPE (PINWORM OVA)

POS. ☐ NEG. ☐

BLOOD

☐ MALARIA

☐ OTHER (Specify)

PARASITOLOGY

This slip will accompany all fecal specimens. Procedures to be followed are:

1. Enter the specimen in the log book. Label the specimen with the log accession number.

2. Perform macroscopic examination. Report:

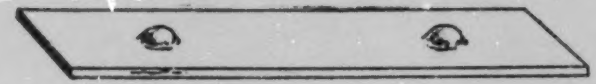
   a. Color

   b. Consistency

   c. Presence of gross blood or mucous, adult worms, proglottids, etc.

3. Perform direct microscopic examination in saline and Lugol's Iodine as follows:

a. On a microscope slide, place side by side a drop of warm physiological saline, and a drop of Lugol's Iodine (3% working solution).



b. With an applicator stick introduce a small portion of feces to the saline drop and mix. Repeat this procedure with the Lugol's drop.

c. Coverslip both drops and examine. First examine the saline side for helminth eggs and larvae, and for trophozoite motility. Next examine the Lugol's side for protozoan cysts.

4. Concentrate the specimen.

LUGOL'S IODINE SOLUTION

1. Stock Solution:

Iodine (powdered crystals)	5 gm.
Potassium Iodide (KI)	10 gm.
Distilled water	100 gm.

Mix ingredients and filter. This stock solution remains satisfactory for months.

2. Working Solution (3% Lugol's)

Dilute stock solution with 5 parts of distilled water. Prepare fresh working solution about every 10 days.

II. Concentration Techniques

A. Zinc Sulfate Centrifugal Flotation

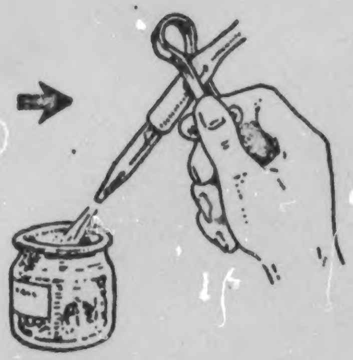
1. Reagent - Zinc Sulfate Solution, Specific Gravity 1.18 - 1.20
    a. Add 331 gm of zinc sulfate (USP) to 1 liter of warm tap water.
    b. Check specific gravity with a hydrometer. Add zinc sulfate or water as needed if specific gravity is below 1.18 or above 1.20.

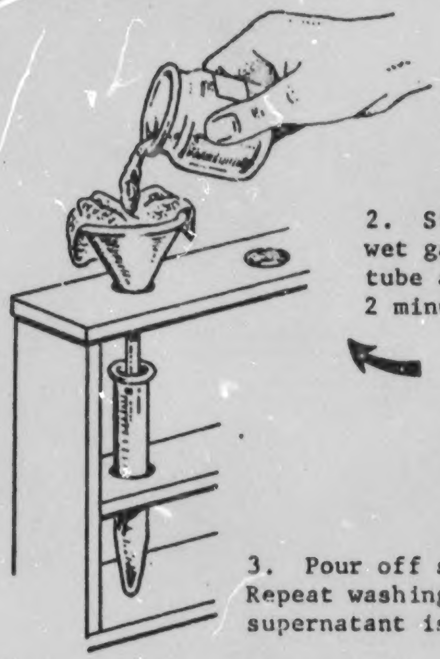
2. Procedure
    a. Place 0.3 to 0.5 gm (1/3 ml to 1/2 ml) of feces in a separate container.
    b. Emulsify in 1 ml of tap water.
    c. Add one 13 x 100 mm test tubefuls of water and mix.
    d. Filter through two layers of wet gauze into a 15 ml centrifuge tube.
    e. Centrifuge for 1 minute @ 2500 RPM.
    f. Pour off supernatant fluid.
    g. Add 1 ml of zinc sulfate solution and mix thoroughly.
    h. Fill tube to 13 or 14 ml mark with zinc sulfate solution.
    i. Centrifuge 1 minute @ 2500 RPM.
    j. Carefully place tube vertically in rack without shaking or spilling.
    k. Gently slide a loop under the surface film and remove 2 or 3 loopfuls of material to glass microscope slide.
    l. Coverslip and examine.

3. Discussion
    a. Advantages
        (1) Easy to perform
        (2) No volatile fluids used
    b. Disadvantages
        (1) Not suitable for fatty stools
        (2) Does not float infertile Ascaris or Trichuris eggs, or the eggs of most Trematodes.
    c. A technical grade of zinc sulfate may be used, but solution must be filtered before use.

B. Formalin-Ether Concentration Technique

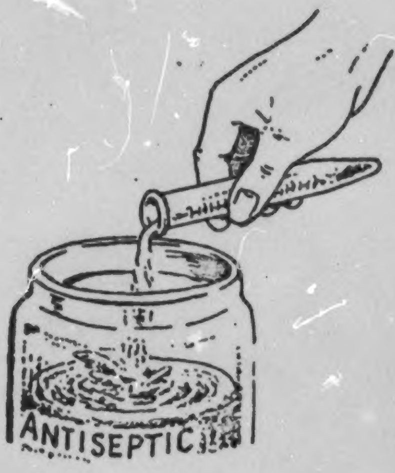
1. After direct examination of the fecal specimen, dilute with water (to destroy the Blastocystics hominis) and mix. NOTE: Alternate method: Add a portion of the specimen to a vial and dilute.



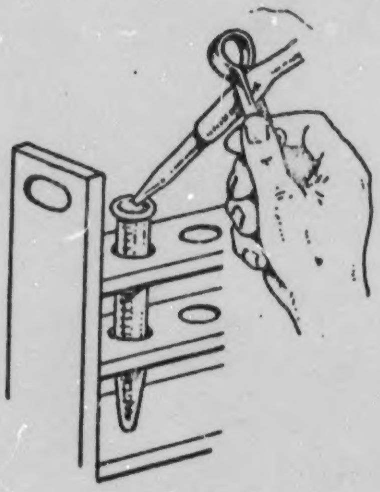


2. Strain the mixture through two layers of wet gauze into a 15 ml graduated centrifuge tube and centrifuge at 1,500 to 2,000 rpm for 2 minutes.

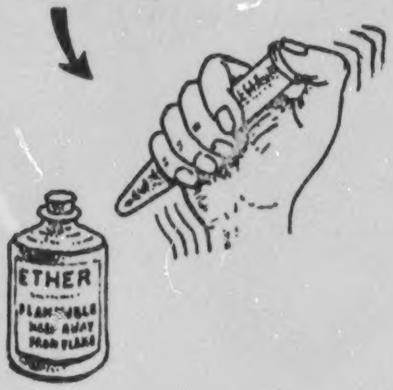
3. Pour off supernatant fluid rapidly. Repeat washing and centrifugation until supernatant is relatively clear.



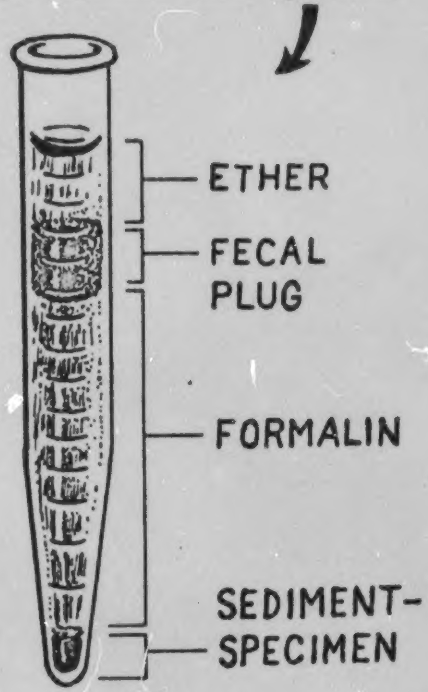
4. Add 7.5 percent formalin to the sediment, mix and allow to stand at least 10 minutes.



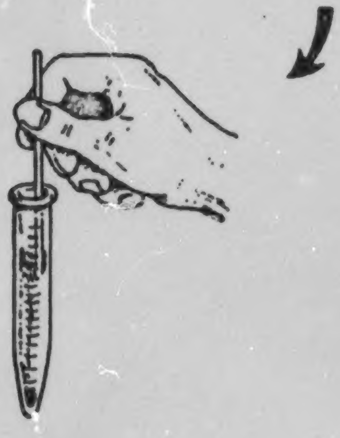
5. Add 3 ml ether to the formalinized specimen and mix thoroughly by shaking vigorously. Recentrifuge



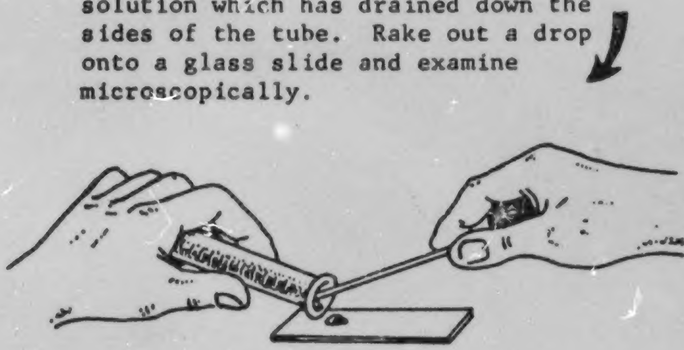
6. Centrifuged formalin-ether specimen.



7. Ream fecal plug. Pour supernatant rapidly into sink with water running. Carefully wipe the sides of the tube with a cotton-tipped applicator stick to remove any oily material and fecal detritus remaining.



8. Mix the sediment at the bottom of the tube with the small amount of solution which has drained down the sides of the tube. Rake out a drop onto a glass slide and examine microscopically.



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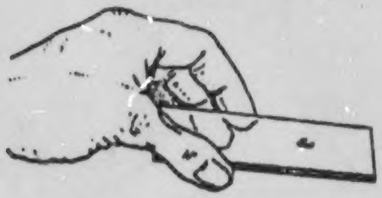
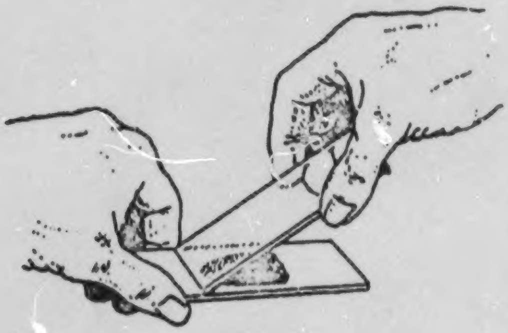
III. Malaria Smears and Giemsa Staining Technique

A. Preparation of the Thick and Thin Smear

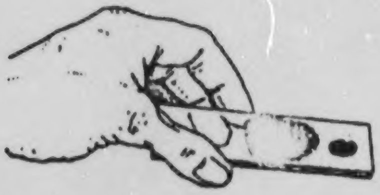
1. After doing the capillary puncture, touch the slide to the blood drop to obtain a small drop of blood on one end of the slide.



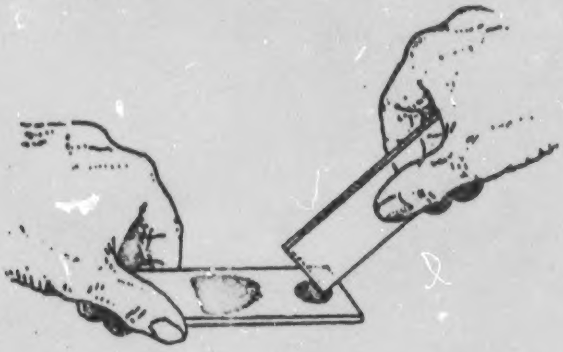
2. Streak the thin smear with a pusher slide.



3. Collect a large drop of blood on the opposite end of the slide.



4. Defibrinate and spread the blood at the thick end to about the size of a dime, using a corner of your "streaking slide". The drop of blood should be spread so thin that news print may be read through it.



5. Allow to dry for at least thirty minutes (depending on the amount of humidity in the atmosphere).

NOTE: Make two slides on new patients. Make at least four slides on all confirmed cases of malaria (for reference specimens).

B. Preparation of Solutions

1. Methylene blue phosphate

Anhydrous methylene blue	1 gm
Na_2HPO_4	3 gm
KH_2PO_4	1 gm

Grind together in a dry mortar. Dissolve 1 gm of mixture into 300 ml of distilled water.

2. Phosphate buffered water

Na_2HPO_4	9.5 gm q. s. to 1,000 ml with distilled water
NaH_2PO_4	9.2 gm q. s. to 1,000 ml with distilled water

Then take the above solutions:

Na_2HPO_4	61 ml	q. s. to 1,000 ml with distilled water
NaH_2PO_4	39 ml	

Prepare from commercial Giemsa buffer salts, 1 gm in 1,000 ml of distilled water, or use the above mixture.

3. Giemsa stain solution

Stock Giemsa stain	1 ml
Phosphate buffered water	49 ml

OR

Giemsa powder	0.75 gm
Methanol	65.00 ml
Glycerol	35.00 ml

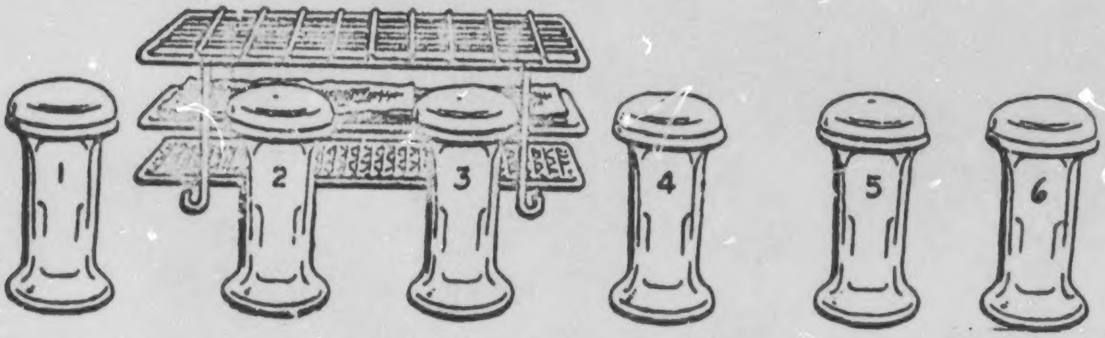
Then take the above solution:

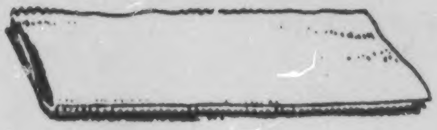
Stain solution	1.00 ml
Phosphate buffered water	49.00 ml

Shake well in a bottle with glass beads, six to ten times daily until well mixed. Filter only when necessary. Store in the dark.

C. Giemsa Stain (modified after Walker)

1. Set up six Coplin jars (or other suitable containers) with the fixing and staining solutions.





2. Fix the thin smear by dipping in methanol for one second and allow to dry. (Do not get the methanol on the thick drop).

3. Dip the thick drop into the tapwater to dehemoglobinize the red blood cells.

4. Remove the slide from the water, touch the end of the slide briefly to a paper towel, and drop the slide into the methylene blue phosphate (mordant) for 1 second.

5. Remove the slide from the methylene blue phosphate, touch the end of the slide briefly to a paper towel, and dip five times into the phosphate buffered water.

6. Touch the end of the slide briefly to a paper towel and place the slide into the Giemsa stain solution for sixty minutes.

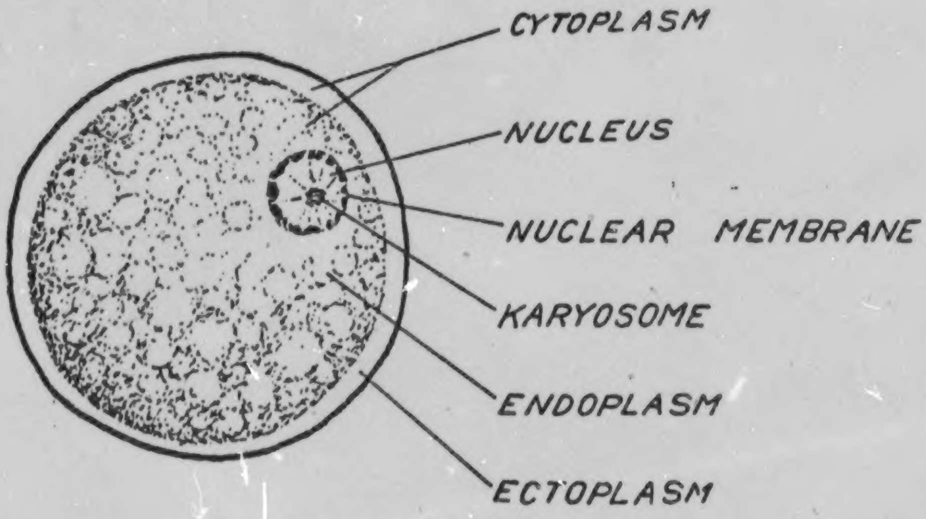
7. After sixty minutes, remove the slide from the Giemsa stain solution, touch the end of the slide briefly to a paper towel, rinse the slide in phosphate buffered water, and place the slide on end in the wire test tube rack to dry.

SECTION C: SUBKINGDOM PROTOZOA

I. Protozoan Cell

Protozoa are all one-cell organisms. Differences reflected in class morphology are mainly in locomotor ectoplasmic organelles (the arrangement of the outer cell structure) for movement or for ingestion of food. Internal differences in nuclear or cytoplasmic substances provide a key to the identification of genus and species.

Protozoan Cell Morphology


CYTOPLASM
NUCLEUS
NUCLEAR MEMBRANE
KARYOSOME
ENDOPLASM
ECTOPLASM

Protozoan Morphological Stages
1. Trophozoite

   a.

   b.

   c.

2. Cyst

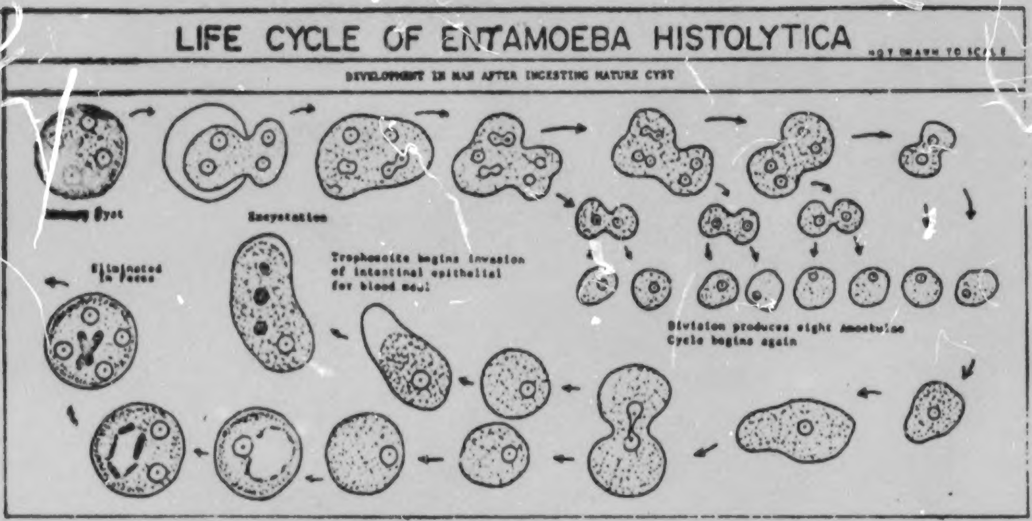
   a.

   b.

   c.

   d.

II. Class Rhizopoda


LIFE CYCLE OF ENTAMOEBA HISTOLYTICA
NOT DRAWN TO SCALE
DEVELOPMENT IN MAN AFTER INGESTING MATURE CYST
Cyst
Excystation
Trophozoite begins invasion of intestinal epithelial for blood meal
Eliminated in Feces
Division produces eight Amoebulae
Cycle begins again

This life cycle is representative of all the members of the class Rhizopoda with the exception of Dientamoeba fragilis.

Amoeba are the medically important members of this class. Amoeba **are characterized** by the ability of the organism to extrude a portion of the cell ectoplasm outward from the cell in an organelle termed a pseudopod or "false foot". Amoeba move by flowing endoplasm in the direction of the pseudopod and engulf food by surrounding it by this same pseudopod process.

General characteristics of the class Rhizopoda:

1. Geographic distribution-

2. Habitat-

3. Specimen-

4. Infective form-

5. Mode of infection-

6.

7.

Subkingdom Protozoa: Class Rhizopoda(continued)

Entamoeba histolytica

(ent-ah-mee-bah hiss-toe-lit-ick-ah)

General Characteristics

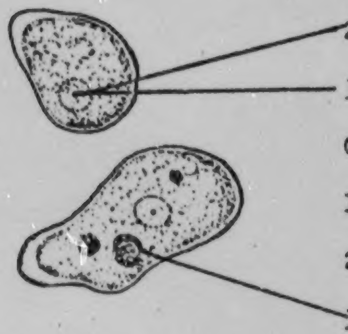
1.

2.

3.

Motility-

Trophozoites (size 15-20 u)



Nucleus

1.

2.

3.

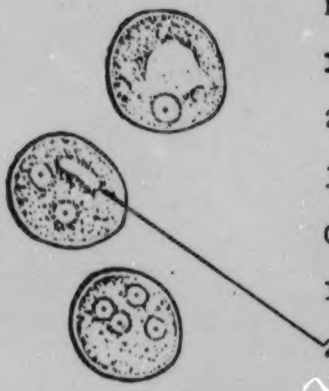
Cytoplasm

1.

2.

3.

Cysts (size 12-15 u)



Nucleus

1.

2.

3.

Cytoplasm

1.

2.

3.

Entamoeba histolytica may inhabit the lumen of the large intestine as a non-pathogen, or may invade tissues of the mucosa, intestinal wall, capillaries, venules, mesentary, peritoneum, liver, lungs, brain, etc. as a pathogen.

Subkingdom Protozoa: Class Rhizopoda(continued)

Entamoeba coli
(ent-ah-mee-bah coal-eye)

General Characteristics

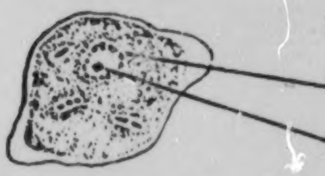
1.

2.

Motility

Trophozoite (size 20-25 u)

Nucleus

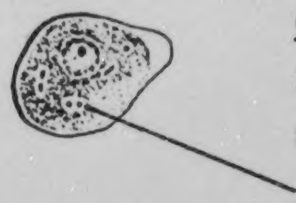


1.

2.

3.

Cytoplasm



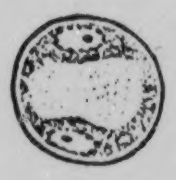
1.

2.

3.

Cyst (size 15-25u)

Nucleus

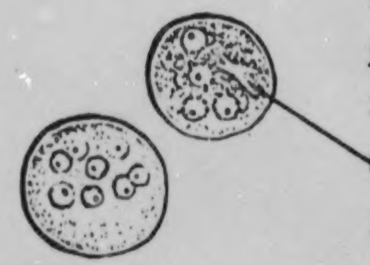


1.

2.

3.

Cytoplasm



1.

2.

3.

Subkingdom Protozoa: Class Rhizopoda(continued)

Endolimax nana
(end-o-lime-ax nah-nah)

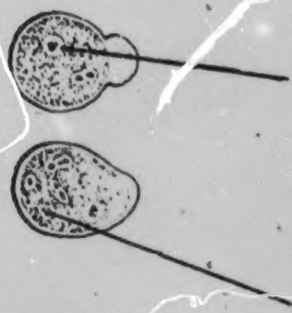
General Characteristics

1.

2.

Motility-

Trophozoite (size 8-10 u)



Nucleus

1.

2.

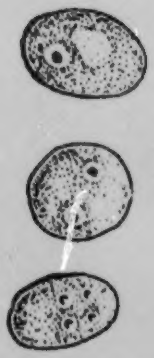
3.

Cytoplasm

1.

2.

Cyst (size 6-8 u)



Nucleus

1.

2.

3.

Cytoplasm

1.

2.

Subkingdom Protozoa: Class Rhizopoda(continued)

Iodamoeba butschlii
(eye-o-dah-mee-bah bush-lee-eye)

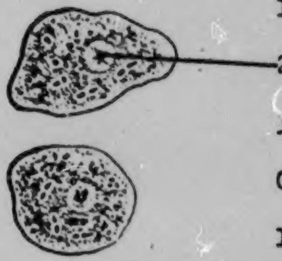
General Characteristics

1.

2.

Motility-

Trophozoite (size 12-15 u)



Nucleus

1.

2.

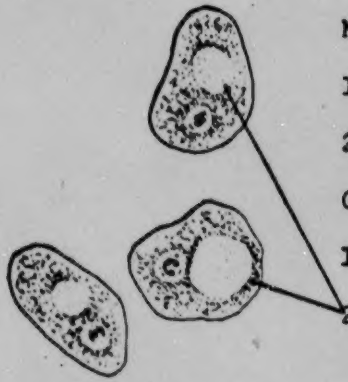
3.

Cytoplasm

1.

2.

Cyst (size 10-12 u)



Nucleus

1.

2.

Cytoplasm

1.

2.

Subkingdom Protozoa: Class Rhizopoda(continued)

Dientamoeba fragilis
(die-ent-ah-mee-bah frah-jill-iss)

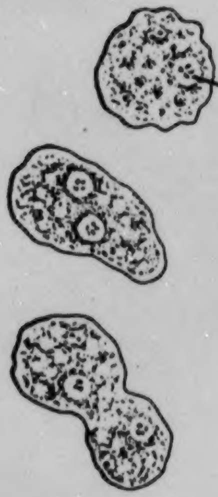
General Characteristics

1.

2.

Motility

Trophozoite (size 9-12u)



Nucleus

1.

2.

3.

Cytoplasm

1.

2.

Cyst - None seen

III. Class Zoomastigophora

Life Cycle of Giardia lamblia

The trophozoites divide by a complicated process of longitudinal binary fission. This involves division of the nuclei, followed by division of the neuromotor apparatus and sucking disc, and finally, the separation of the cytoplasm. This completes the formation of two daughter trophozoites.

In preparation for encystment the trophozoites retract their flagella into the axostyles; the cytoplasm becomes condensed, and a thin, tough hyaline membrane (cyst wall) is secreted. Recently formed cysts have two nuclei, and mature cysts have four nuclei. These are situated at one end of the cyst until the organism is ready to divide to form two individuals. As the cyst ripens the internal structures and the sucking disc are doubled, so that when excystation occurs all that is required for the production of identical twins is the division of the cytoplasm and the growth of flagella from the axostyles.

The members of the class Zoomastigophora are commonly referred to as "flagellates" and are characterized by a whip-like organelle called a flagellum. The lashing action of the flagellum (plural: flagella) produces movement or directs food particles to the cytostome (primitive mouth) for ingestion.

General Characteristics of the Class Zoomastigophora

1.

2.

3.

Subkingdom Protozoa: Class Zoomastigophora (continued)

Giardia lamblia
(gee-are-dee-ah lamb-lee-ah)

General Characteristics

1.

2.

3. Habitat-

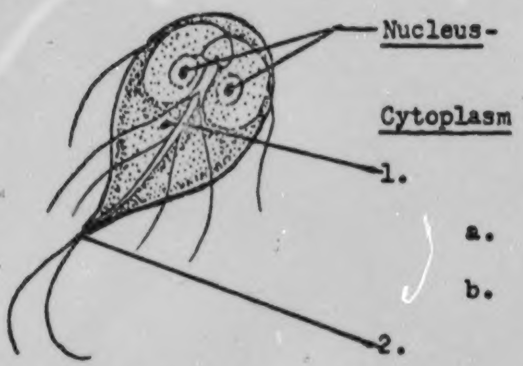
4. Specimen-

5. Infective form-

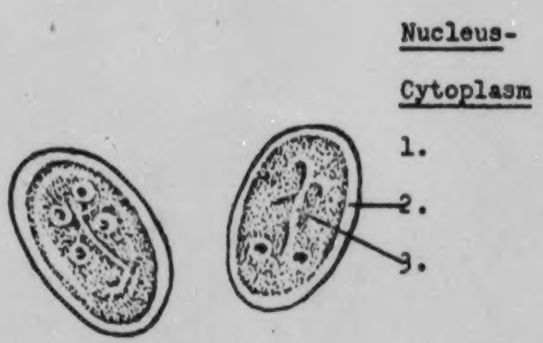
6. Mode of infection-

Motility

Trophozoite (size 12-15u)


Nucleus-
Cytoplasm
1.
a.
b.
2.

Cyst (size 11-12u)


Nucleus-
Cytoplasm
1.
2.
3.

Subkingdom Protozoa: Class Zoomastigophora (continued)

Trichomonas vaginalis
(trick-o-mow-nass vaj-in-al-iss)

General Characteristics

1.

2. Habitat-

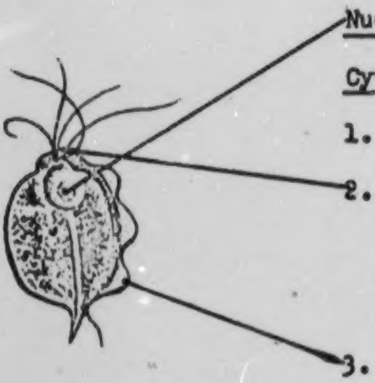
3. Specimen-

4. Infective form-

5. Mode of infection-

Motility-

Trophozoite (size 12-14u)



Nucleus-

Cytoplasm

1.

2.

a.

b.

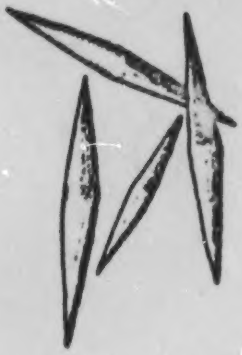
3.

Cyst - None seen

NOTE: Other species of Trichomonas are determined by specimen source.

1. Trichomonas tenax-
2. Trichomonas hominis-

IV. Other Findings



Charcot-Leyden crystals (size 7-15u)

1.

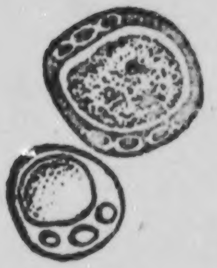
2.

  a.

  b.

  c.

3.



Blastocystis hominis (size 7-8u)

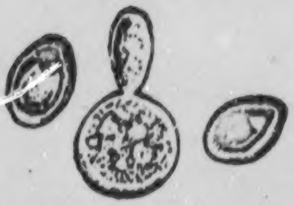
1.

2.

  a.

  b.

  c.



Other yeast (size less than 7u)

1.

2.

  a.

  b.

Subkingdom Protozoa: Other Findings (continued)

Cellular Elements from Intestinal Tract

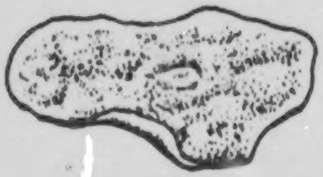
1.

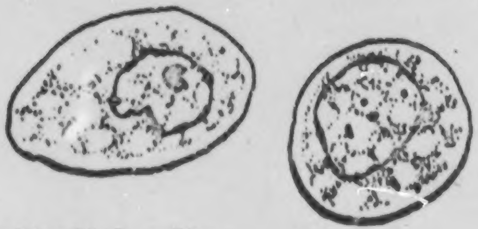
2.

a.

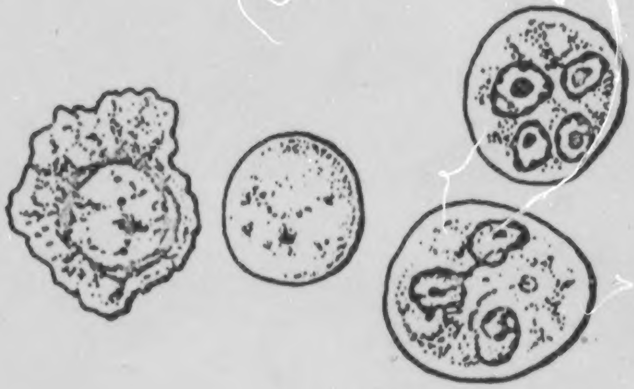
b.

c.



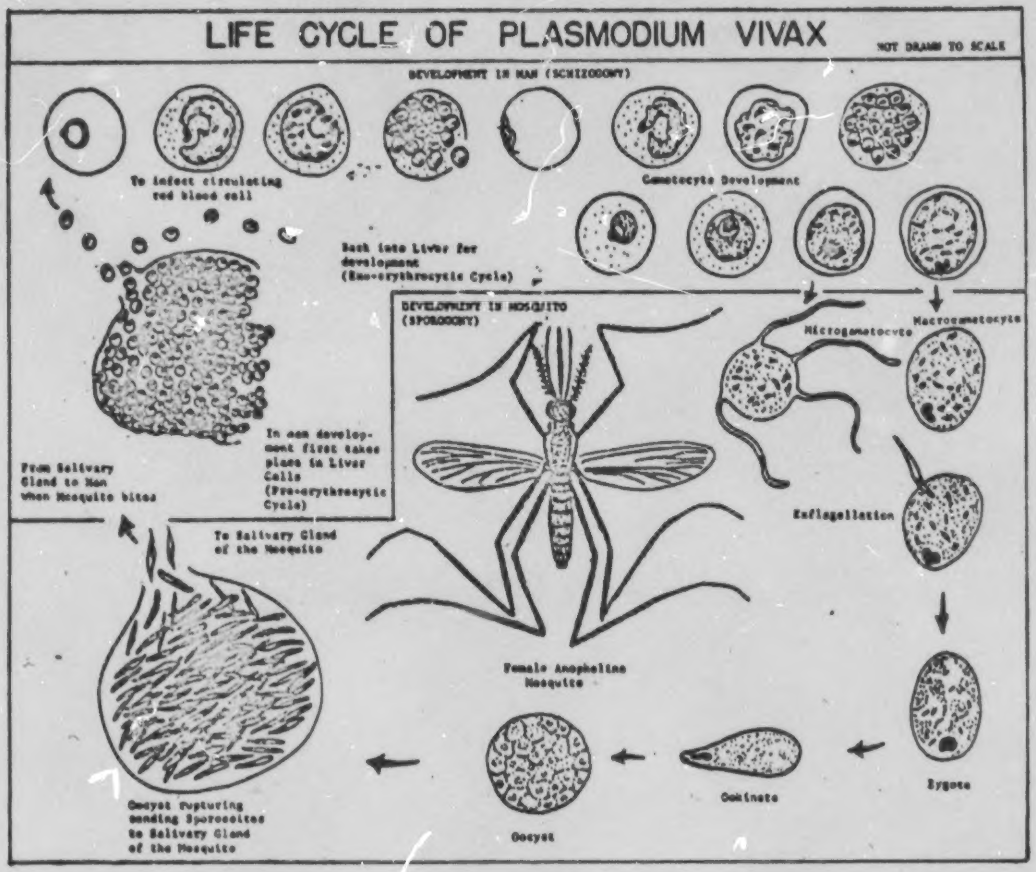


Epithelial cells



Leukocytes

V. Class Telosporea


LIFE CYCLE OF PLASMODIUM VIVAX
NOT DRAWN TO SCALE
DEVELOPMENT IN MAN (SCHIZOGONY)
To infect circulating red blood cell
Gametocyte Development
Back into Liver for development (Exo-erythrocytic Cycle)
In man development first takes place in Liver Cells (Pre-erythrocytic Cycle)
From Salivary Gland to Man when Mosquito bites
DEVELOPMENT IN MOSQUITO (SPOROGONY)
Microgametocyte
Macrogametocyte
Exflagellation
To Salivary Gland of the Mosquito
Female Anopheline Mosquito
Zygote
Ookinete
Oocyst
Oocyst rupturing sending Sporozoites to Salivary Gland of the Mosquito

Species of Plasmodium in Man

1.

2.

3.

4.

General Characteristics of Class Telosporea

1.

2.

3.

Subkingdon Protozoa: Class Telosporea (continued)

Hosts

1.

2.

Morphological Forms Occurring in Man

1.

2.

  a.

  b.

3.

  a.

  b.

  c.

4.

  a.

    (1)

    (2)

  b.

Subkingdom Protozoa: Class Telosporea (continued)

Plasmodium vivax
(Plaz-moe-dee-um vie-vax)

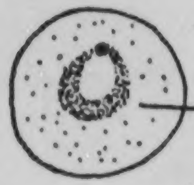
Common name-

Geographic distribution

1.

2.

Fever-chill cycle-

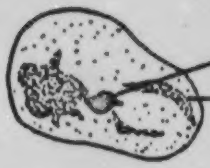


Infected RBC

1.

2.

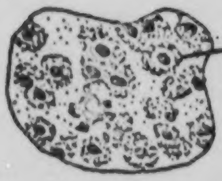
3.



Mature Trophozoite

1.

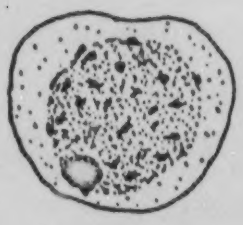
2.



Schizont

1.

2.



Gametocyte

1.

2.

Subkingdom Protozoa: Class Telosporea (continued)

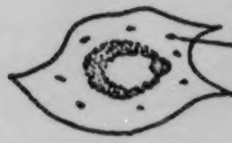
Plasmodium ovale
(Plaz-moe-dee-um oh-vah-lee)

Common name-

Geographic distribution-

Fever-chill cycle-

Infected RBC



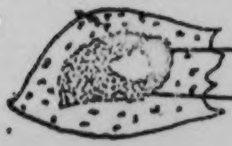
1.

2.

3.

4.

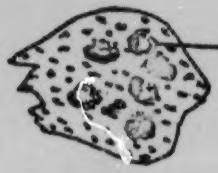
Mature Trophozoite



1.

2.

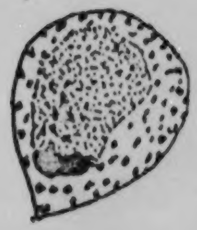
Schizont



1.

2.

Gametocyte



1.

2.

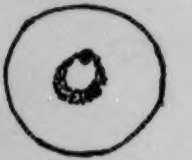
Subkingdom Protozoa: Class Telosporea (continued)

Plasmodium malariae
(Plaz-moe-dee-um mal-air-ee-yeah)

Common name-

Geographic distribution-

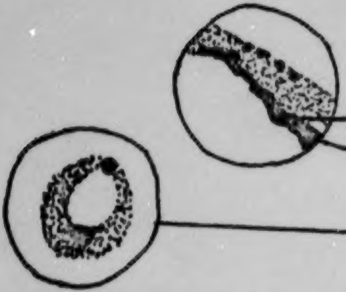
Fever-chill cycle-



Infected RBC

1.

2.



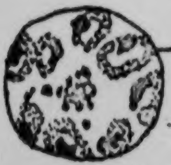
Mature Trophozoite

1.

2.

3.

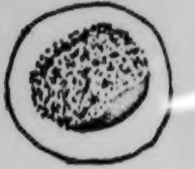
4.



Schizont

1.

2.



Gametocyte

1.

2.

Subkingdom Protozoa: Class Telosporea (continued)

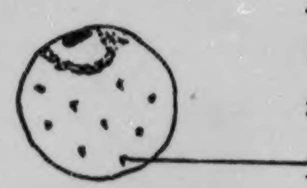
Plasmodium falciparum
(Plaz-moe-dee-um fal-sip-are-um)

Common name-

Geographic distribution-

Fever-chill cycle-

Infected RBC

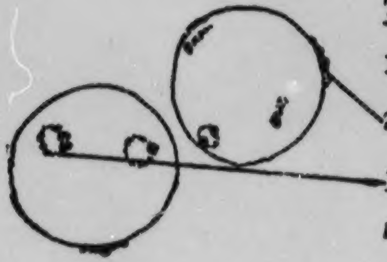


1.

2.

3.

Trophozoite



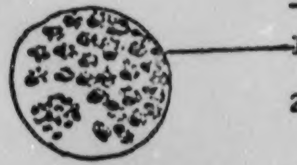
1.

2.

3.

4.

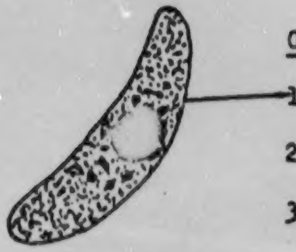
Schizont



1.

2.

Gametocyte



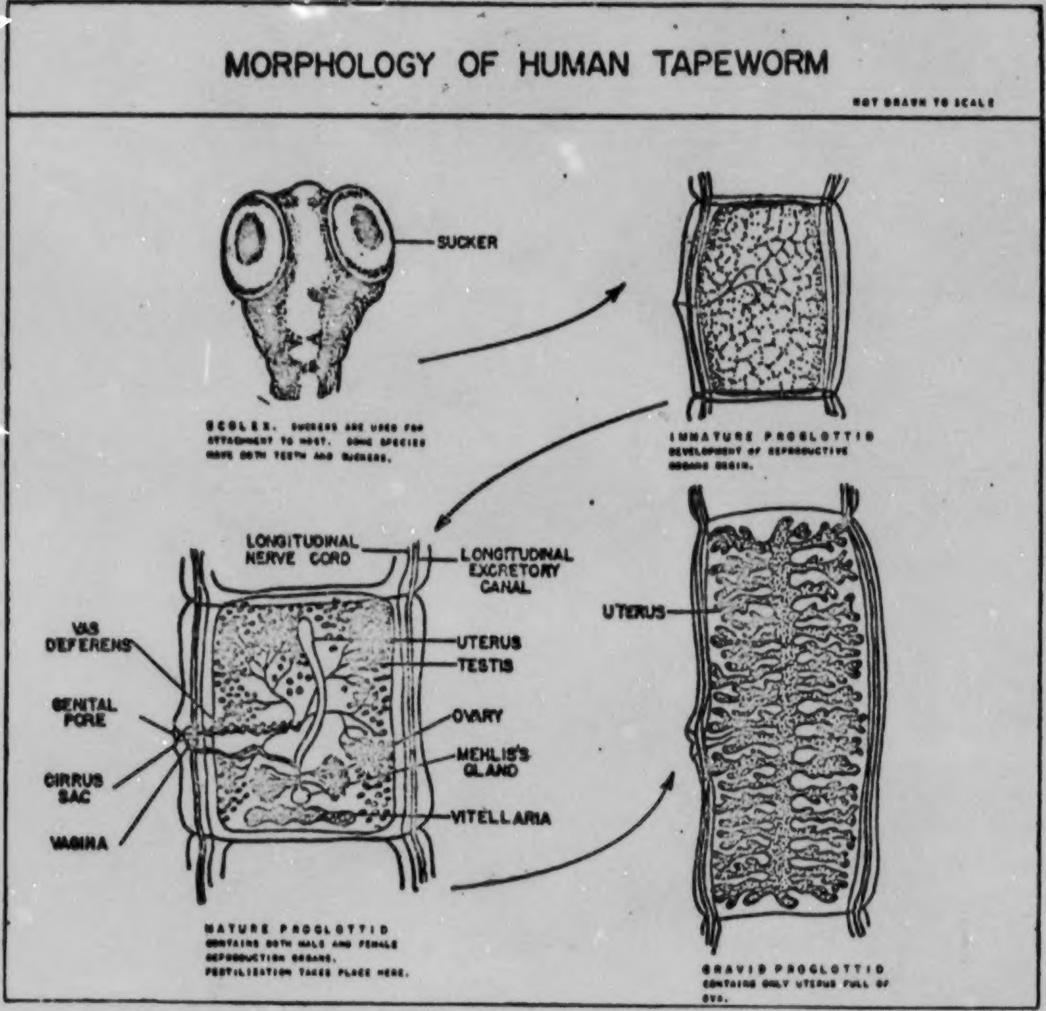
1.

2.

3.

SECTION D: SUBKINGDOM METAZOA

I. Class Cestoda



MORPHOLOGY OF HUMAN TAPEWORM

NOT DRAWN TO SCALE

SCOLEX. SUCKERS ARE USED FOR ATTACHMENT TO HOST. SOME SPECIES HAVE BOTH TEETH AND SUCKERS.

IMMATURE PROGLOTTID DEVELOPMENT OF REPRODUCTIVE ORGANS BEGIN.

MATURE PROGLOTTID CONTAINS BOTH MALE AND FEMALE REPRODUCTION ORGANS. FERTILIZATION TAKES PLACE HERE.

GRAVID PROGLOTTID CONTAINS ONLY UTERUS FULL OF OVA.

SUCKER
LONGITUDINAL NERVE CORD
LONGITUDINAL EXCRETORY CANAL
VAS DEFERENS
GENITAL PORE
CIRRUS SAC
VAGINA
UTERUS
TESTIS
OVARY
MEHLIS'S GLAND
VITELLARIA
UTERUS

General Characteristics of Class Cestoda

1.

2.

3.

4.

212

Subkingdom Metazoa: Class Cestoda (continued)

5.

a.

(1)

(2)

b.

c.

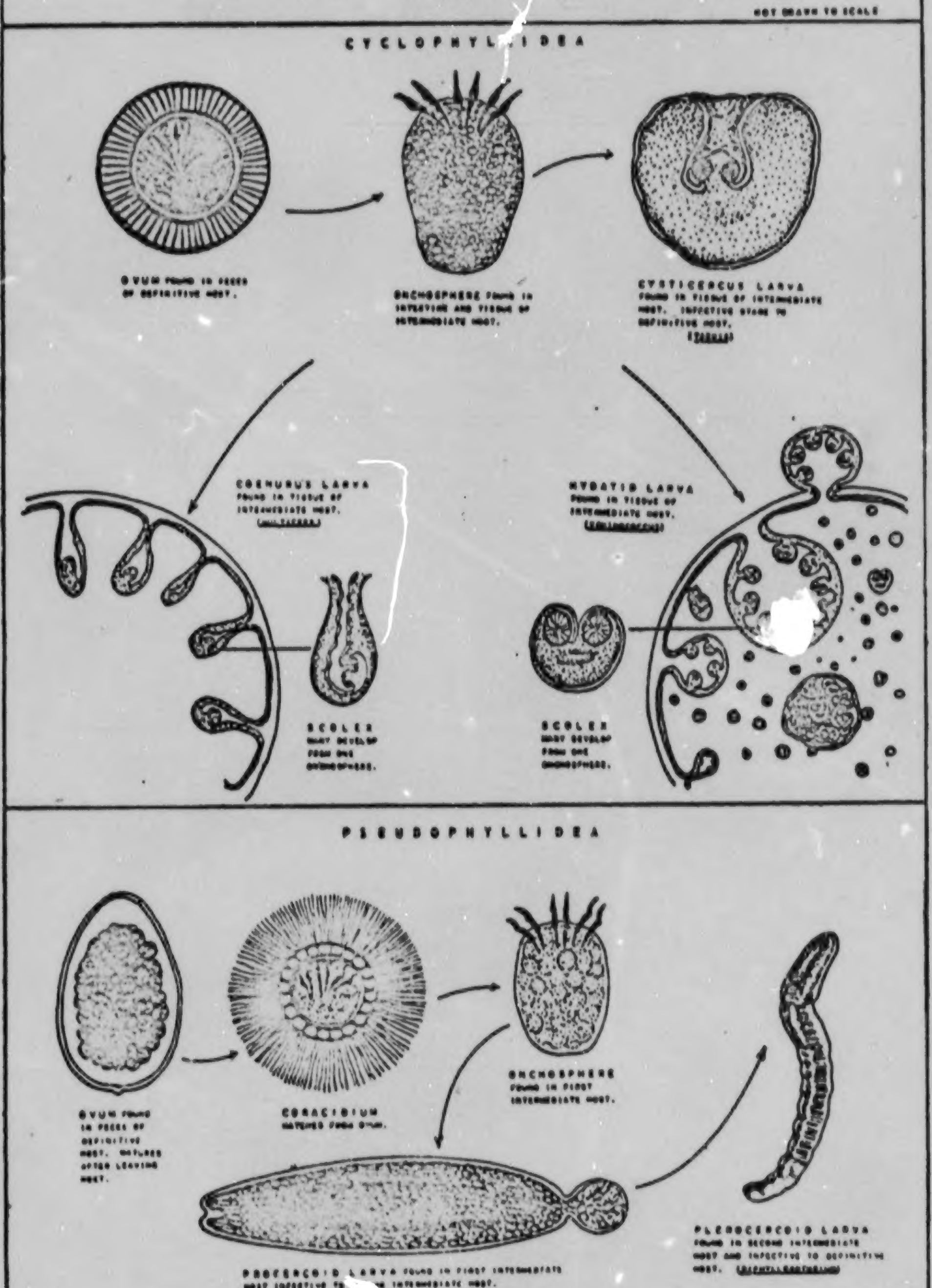
(1)

(2)

(3)

Subkingdom Metazoa: Class Cestoda (continued)

LIFE CYCLES OF HUMAN TAPEWORMS


NOT DRAWN TO SCALE
CYCLOPHYLLIDEA
OVUM FOUND IN FECES OF DEFINITIVE HOST.
ONCHOSPHERE FOUND IN INTESTINE AND TISSUE OF INTERMEDIATE HOST.
CYSTICERCUS LARVA FOUND IN TISSUE OF INTERMEDIATE HOST. INFECTIVE STAGE TO DEFINITIVE HOST.
COENURUS LARVA FOUND IN TISSUE OF INTERMEDIATE HOST.
HYDATID LARVA FOUND IN TISSUE OF INTERMEDIATE HOST.
SCOLEX MAY DEVELOP FROM ONE ONCHOSPHERE.
SCOLEX MANY DEVELOP FROM ONE ONCHOSPHERE.
PSEUDOPHYLLIDEA
OVUM FOUND IN FECES OF DEFINITIVE HOST. MATURES AFTER LEAVING HOST.
CORACIDIUM HATCHED FROM OVUM.
ONCHOSPHERE FOUND IN FIRST INTERMEDIATE HOST.
PROCERCOID LARVA FOUND IN FIRST INTERMEDIATE HOST INFECTIVE TO [illegible] INTERMEDIATE HOST.
PLEROCERCOID LARVA FOUND IN SECOND INTERMEDIATE HOST AND INFECTIVE TO DEFINITIVE HOST.

Subkingdom Metazoa: Class Cestoda (continued)

Taenia solium
(Tay-knee-ah soul-ee-um)

Common name -

Geographic distribution-

Habitat-

Specimen-

Infective form-

Mode of infection-

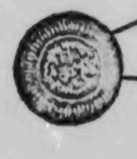
Intermediate host

1.

2.

Identification forms

Egg (size 31-43u)



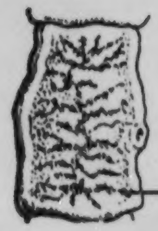
1.

2.

3.

4.

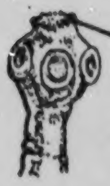
5.



Gravid proglottid

1.

2.



Scolex -

Subkingdom Metazoa: Class Cestoda (continued)

Taenia saginata
(Tay-knee-ah sah-gin-ah-tah)

Common name-

Geographic distribution-

Habitat

Specimen

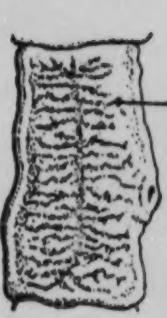
Infective form

Mode of infection-

Intermediate host-

Identification forms

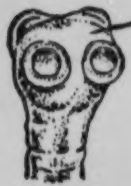
Egg - same as *Taenia solium*



Gravid proglottid

1.

2.



Scolex -

Subkingdom Metazoa: Class Cestoda (continued)

Hymenolepis nana
(High-men-nell-lip-piss nah-nah)

Common name-

Geographic distribution-

Habitat-

Specimen-

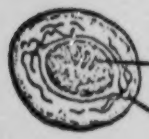
Infective form-

Mode of infection-

Autoinfective

Identification forms

Egg (size 37- 47u)



1.

2.

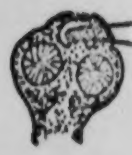
3.

4.

5.

Gravid proglottid -

Scolex



1.

2.

Subkingdom Metazoa: Class Cestoda (continued)

Diphyllobothrium latum
(Die-fill-oh-baa-three-um lay-tum)

Common name-

Geographic distribution-

Habitat-

Specimen-

Infective form-

Mode of infection-

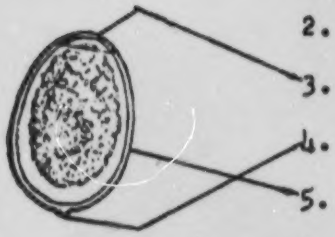
Intermediate hosts

1.

2.

Identification forms

Egg (size 66 x 44u)



1.

2.

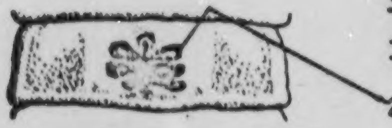
3.

4.

5.

6.

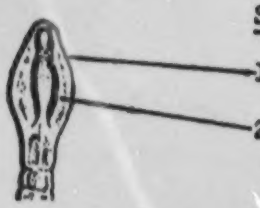
Gravid proglottid



1.

2.

Scolex

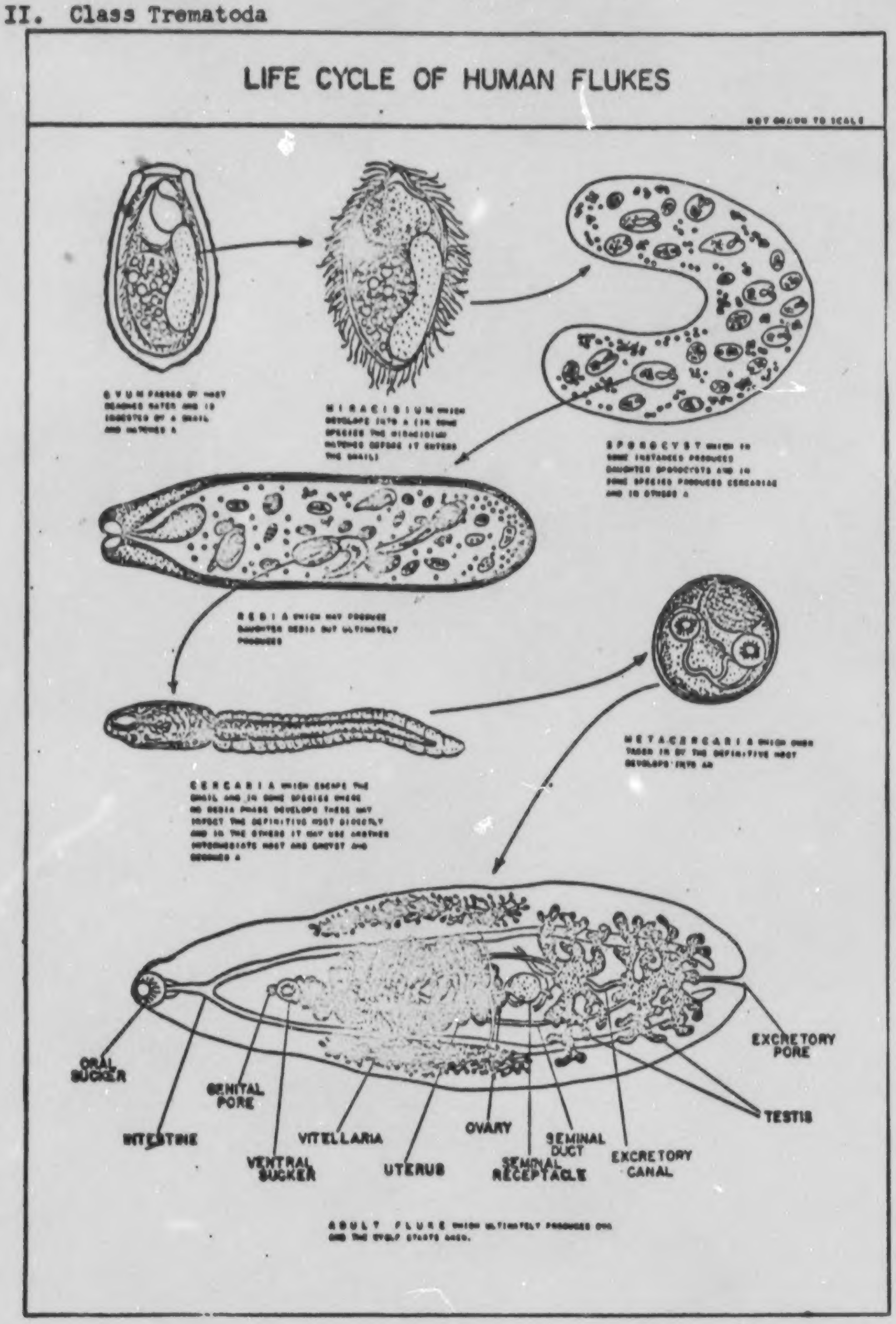


1.

2.

II. Class Trematoda

LIFE CYCLE OF HUMAN FLUKES


NOT DRAWN TO SCALE
OVUM PASSED BY HOST REACHES WATER AND IS INGESTED BY A SNAIL AND HATCHES A
MIRACIDIUM WHICH DEVELOPS INTO A (IN SOME SPECIES THE MIRACIDIUM HATCHES BEFORE IT ENTERS THE SNAIL)
SPOROCYST WHICH IN SOME INSTANCES PRODUCES DAUGHTER SPOROCYSTS AND IN SOME SPECIES PRODUCES CERCARIAE AND IN OTHERS A
REDIA WHICH MAY PRODUCE DAUGHTER REDIA BUT ULTIMATELY PRODUCES
CERCARIA WHICH ESCAPE THE SNAIL AND IN SOME SPECIES WHERE NO REDIA PHASE DEVELOPS THESE MAY INFECT THE DEFINITIVE HOST DIRECTLY AND IN THE OTHERS IT MAY USE ANOTHER INTERMEDIATE HOST AND ENCYST AND BECOMES A
METACERCARIA WHICH WHEN TAKEN IN BY THE DEFINITIVE HOST DEVELOPS INTO AN
ORAL SUCKER
INTESTINE
GENITAL PORE
VENTRAL SUCKER
VITELLARIA
UTERUS
OVARY
SEMINAL RECEPTACLE
SEMINAL DUCT
EXCRETORY CANAL
TESTIS
EXCRETORY PORE
ADULT FLUKE WHICH ULTIMATELY PRODUCES OVA AND THE CYCLE STARTS AGAIN.

Subkingdom Metazoa: Class Trematoda (continued)

General Characteristics of Class Trematoda

1.

2.

3.

    a.

    b.

4.

General Characteristics of Monecious Trematodes

1.

2.

    a.

    b.

3.

4.

Subkingdom Metazoa: Class Trematoda (continued)

Paragonimus westermani
(Pair-ah-gone-im-us west-er-mon-ee)

Common name-

Geographic distribution-

Habitat-

Specimen-

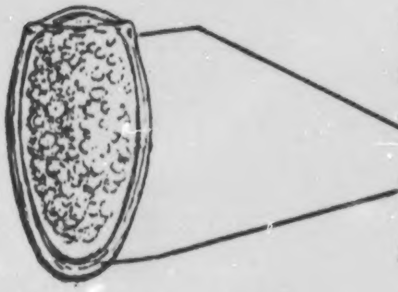
Intermediate hosts

1.

2.

Identification form

Egg (size 95 x 55u)



1.

2.

3.

4.

5.

Subkingdom Metazoa: Class Trematoda (continued)

Clonorchis sinensis
(Klō-nor-kiss sin-en-sis)

Common name-

Geographic distribution-

Habitat-

Specimen-

Intermediate hosts

1.

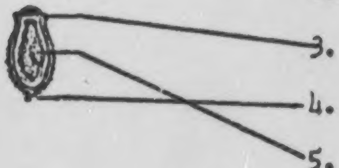
2.

Identification form

Egg (size 29 x 16u)

1.

2.



3.

4.

5.

Similar eggs

1.

2.

3.

222

Subkingdom Metazoa: Class Trematoda (continued)

Fasciola hepatica
(Fass-ee-oh-lah hep-attic-ah)

Common name-

Geographic distribution-

Habitat-

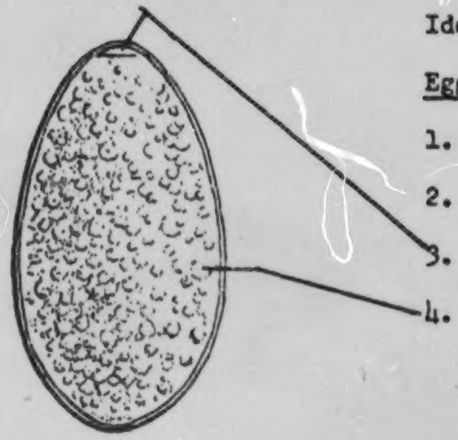
Specimen-

Intermediate hosts

1.

2.

Reservoir host-



Identification form

Egg (size 140 x 80u)

1.

2.

3.

4.

Similar egg-

Subkingdom Metazoa: Class Trematoda (continued)

General Characteristics of Diecious Trematodes

1.
2.
3.
4.
5.

Schistosoma mansoni
(Shis-toe-so-mah man-so-nī)

Common name-

Geographic distribution-

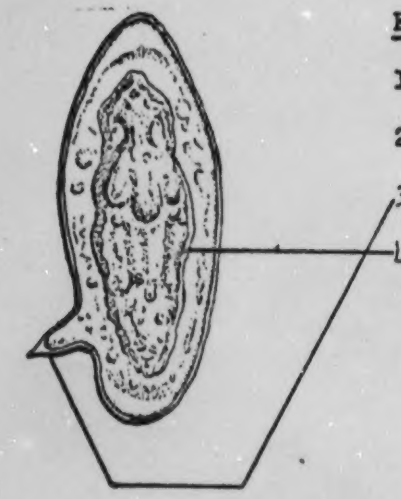
Habitat-

Specimen-

Identification form

Egg (size 155 x 65u)

1.
2.
3.
4.



Subkingdom Metazoa: Class Trematoda (continued)

Schistosoma japonicum
(Shis-toe-so-mah jap on-i-come)

Common name-

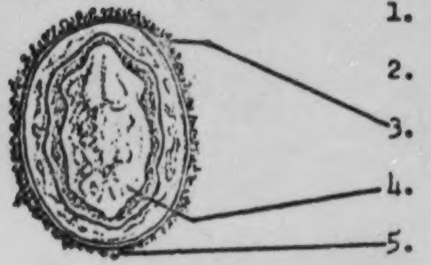
Geographic distribution-

Habitat-

Specimen-

Identification form

Egg (size 85 x 60u)



Subkingdom Metazoa: Class Trematoda (continued)

Schistosoma haematobium
(Shis-toe-so-mah hēma-toe-bee-um)

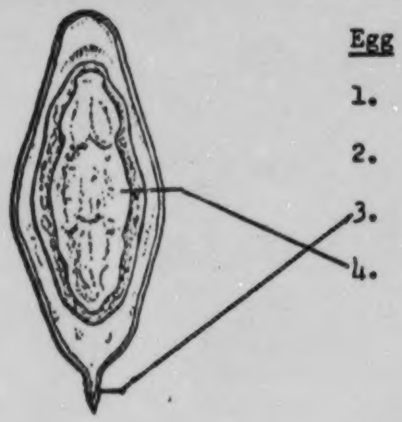
Common name-

Geographic distribution-

Habitat-

Specimen-

Identification form



Egg (size 150 x 60u)

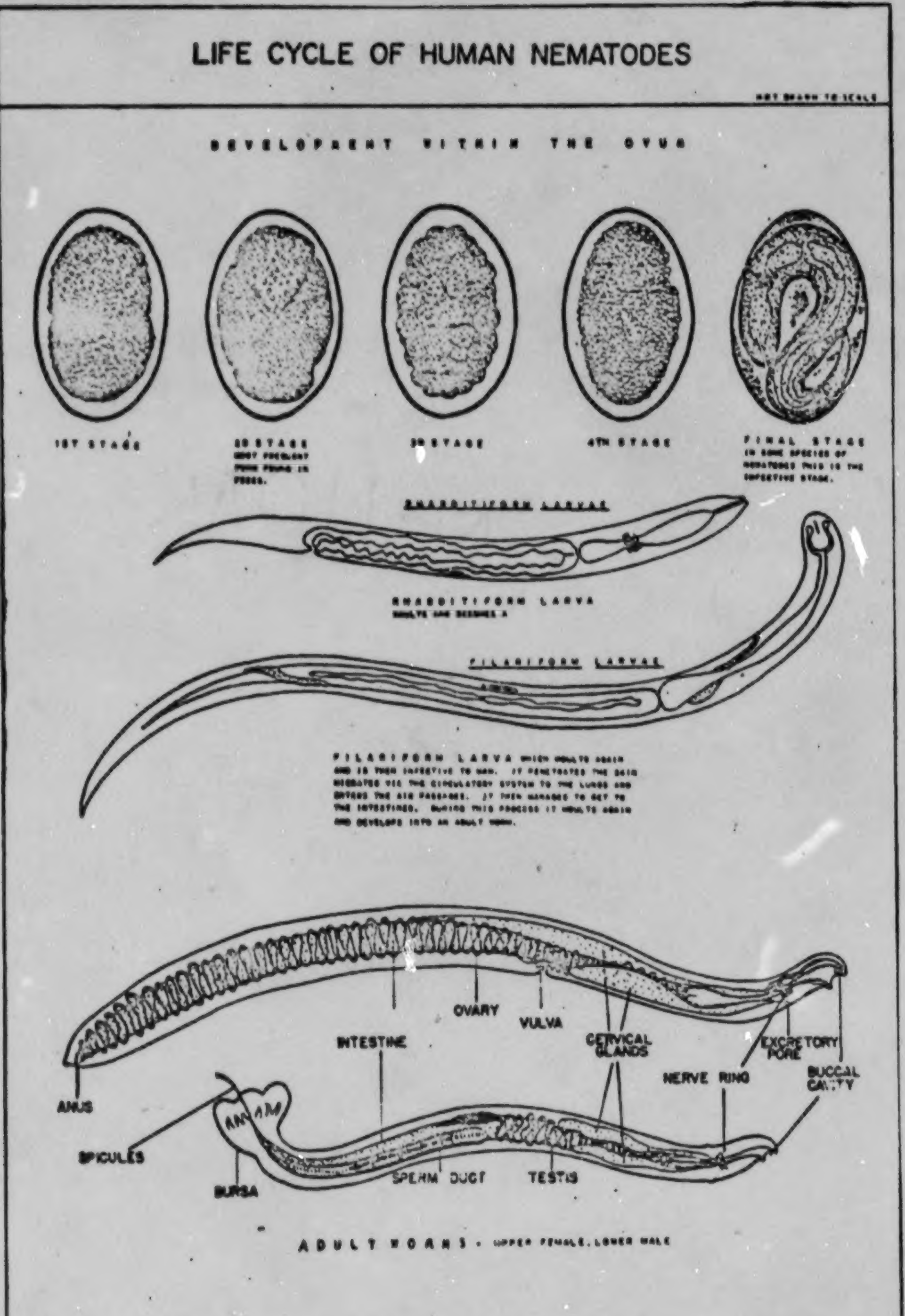
1.

2.

3.

4.

III. Class Nematoda



LIFE CYCLE OF HUMAN NEMATODES

NOT DRAWN TO SCALE

DEVELOPMENT WITHIN THE OVUM
1ST STAGE
2D STAGE MOST PREVALENT FORM FOUND IN FECES.
3D STAGE
4TH STAGE
FINAL STAGE IN SOME SPECIES OF NEMATODES THIS IS THE INFECTIVE STAGE.
RHABDITIFORM LARVAE
RHABDITIFORM LARVA MOULTS AND BECOMES A
FILARIFORM LARVAE
OVARY
VULVA
INTESTINE
CERVICAL GLANDS
EXCRETORY PORE
NERVE RING
BUCCAL CAVITY
ANUS
SPICULES
BURSA
SPERM DUCT
TESTIS

FILARIFORM LARVA WHICH MOULTS AGAIN AND IS THEN INFECTIVE TO MAN. IT PENETRATES THE SKIN MIGRATES VIA THE CIRCULATORY SYSTEM TO THE LUNGS AND ENTERS THE AIR PASSAGES. IT THEN MANAGES TO GET TO THE INTESTINES. DURING THIS PROCESS IT MOULTS AGAIN AND DEVELOPS INTO AN ADULT WORM.

ADULT WORMS - UPPER FEMALE, LOWER MALE

Subkingdom Metazoa: Class Nematoda (continued)

General Characteristics of Class Nematoda

1.
2.
    a.
    b.
3.

Subkingdom Metazoa: Class Nematoda (continued)

Nematodes Acquired by Ingestion of the Infective Form

Enterobius vermicularis
(Enter-oh-bee-us ver-mick-yule-air-iss)

Common name-

Geographic distribution-

Habitat-

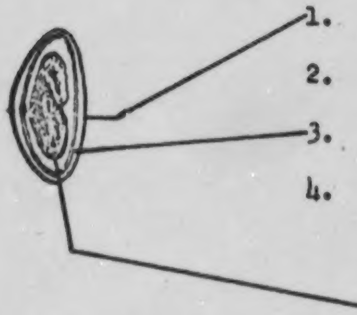
Specimen-

Infective form-

Autoinfective

Identification forms

Egg (size 55 x 26u)



1.

2.

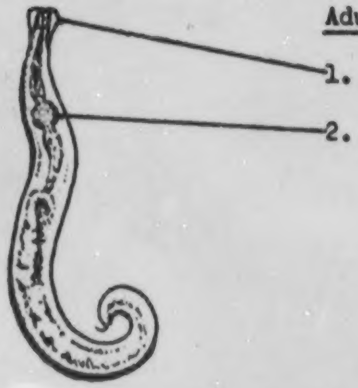
3.

4.

a.

b.

Adult



1.

2.

Subkingdom Metazoa: Class Nematoda (continued)

Ascaris lumbricoides
(Ask-air-iss lum-brick-oiy-dees)

Common name-

Geographic distribution-

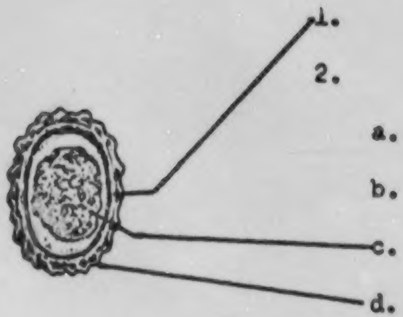
Habitat-

Specimen-

Infective form-

Identification form

Egg (size 60 x 45u)



1.

2.

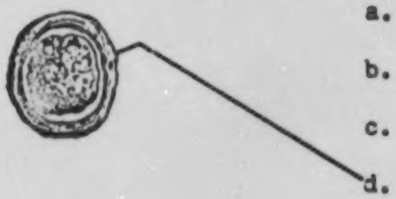
a.

b.

c.

d.

3.



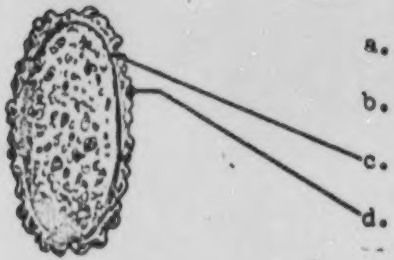
a.

b.

c.

d.

4.



a.

b.

c.

d.

5.

Subkingdom Metazoa: Class Nematoda (continued)

Trichuris trichiura
(Trick-your-iss tricky-your-ah)

Common name-

Geographic distribution-

Habitat-

Specimen-

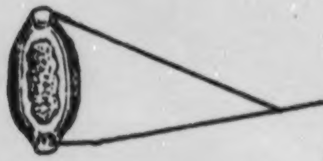
Infective form-

Identification forms

Egg (size 54 x 22u)

1.

2.



3.

4.

Adult-

Subkingdom Metazoa: Class Nematoda (continued)

Nematodes Acquired by Active Penetration of the Infective Form

Hookworm species

Ancylostoma duodenale (An-sill-lost-um-ah dew-odd-en-alley)	*Necator americanus* (Neck-ā-tore am-air-ick-ān-us)

Common name-

Geographic distribution-

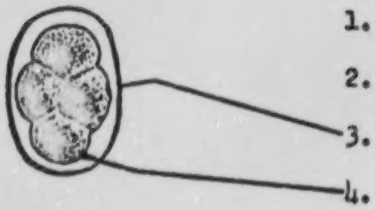
Habitat-

Specimen-

Infective form-

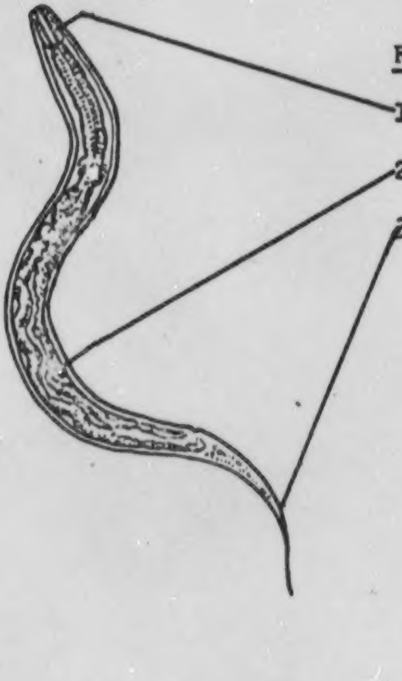
Identification forms

Egg (size 60 x 40u)



1.
2.
3.
4.

Rhabditiform larva (size 250 x 17u)



1.
2.
3.

Subkingdom Metazoa: Class Nematoda (continued)

Strongyloides stercoralis
(Stron-je-loy-deez stare-core-alice)

Common name-

Geographic distribution-

Habitat-

Specimen-

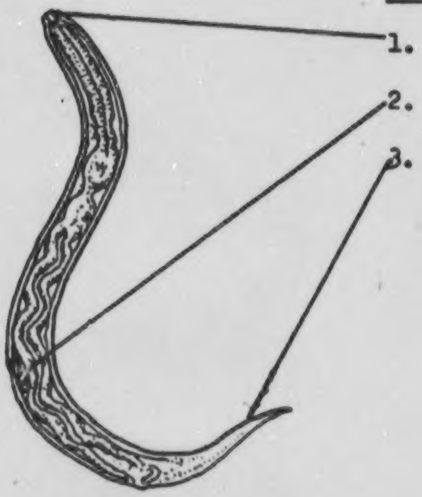
Infective form-

Autoinfective

Identification forms

Egg-

Rhabditiform larva (size 225 x 16u)



SECTION E: OTHER PARASITES

A. Naegleria species

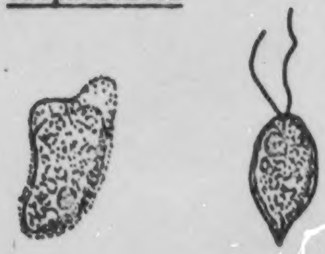
General characteristics

1.

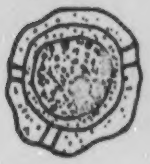
2.

3.

Trophozoites



Cyst



Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Laboratory identification

1.

- a.
- b.

2.

234

B. Balantidium coli

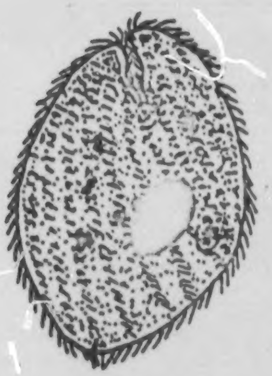
General characteristics

1.

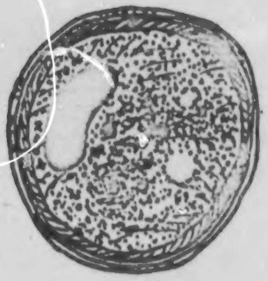
2.

3.

Trophozoite



Cyst



Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Disease

1.

2.

Laboratory identification-

C. Toxoplasma gondii

General characterisitcs

1.

2.

Geographic distribution-

Habitat-

Specimen-

Mode of infection

1.

2.

Disease-

1.

2.

Laboratory identification

1.

2.

236

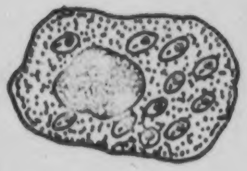
D. Leishmania species

General characteristics

1.

2.

3.



4.

5.

6. Vector-

7. Reservoir hosts-

Leishmania donovani

Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Disease-

1.

2.

Other Parasites: Leishmania species (continued)

Leishmania tropica

Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Disease-

1.

2.

Leishmania braziliensis

Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Disease-

1.

2.

Laboratory identification

1.

2.

3.

E. *Trypanosoma* species

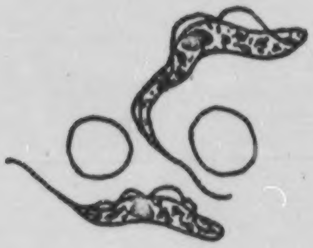
General characteristics

1.

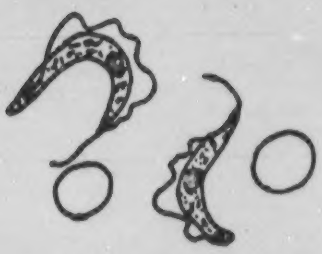
2.

   a.

   b.



Trypanosoma gambiense
Trypanosoma rhodesiense



Trypanosoma cruzi

Trypanosoma gambiense and *Trypanosoma rhodesiense*

Geographic distribution-

1.

2.

Habitat-

Specimen-

Mode of infection-

Disease

1.

2.

Other Parasites: Trypanosoma species (continued)

Trypanosoma cruzi

Geographic distribution-

Habitat-

Specimen-

Mode of infection-

Disease-

Laboratory Identification

1.

2.

3.

4.

240

F. *Echinococcus* species

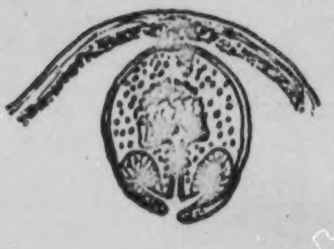
General characteristics

1.

2.

3.

4.



Geographic distribution-

Habitat

1.

2.

Mode of infection-

Specimen-

Diseases-

1.

2.

Laboratory identification

1.

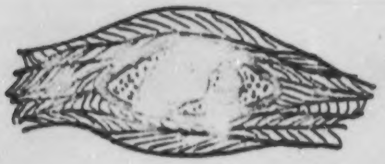
2.

G. **Trichinella spiralis**

General characteristics

1.

2.



Geographic distribution-

Habitat

1.

2.

Specimen-

Mode of infection-

Disease-

Laboratory identification

1.

2.

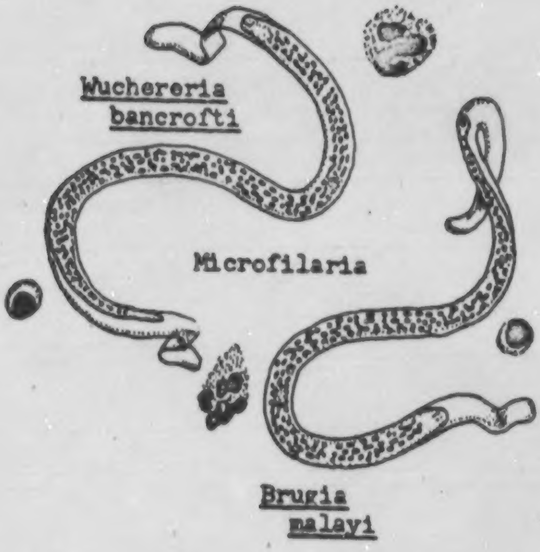
H. Filarial Worms

General characteristics

1.

2.

3.


Wuchereria bancrofti
Microfilaria
Brugia malayi

Geographic distribution of common species

1. *Wuchereria bancrofti*-

2. *Brugia malayi*-

Habitat

1.

2.

Specimen-

Disease-

Laboratory identification

1.

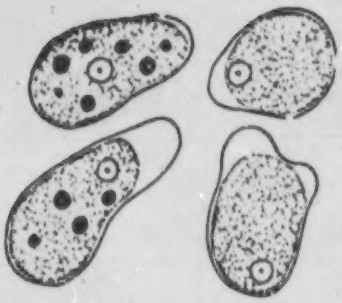
2.

SECTION F: ANNEX

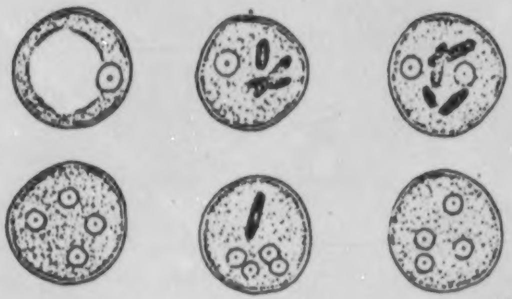
Identification Forms of Class Rhizopoda

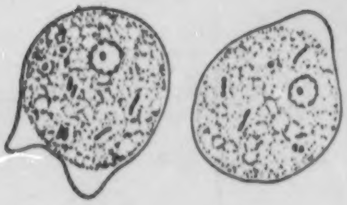
Trophozoites

Cysts

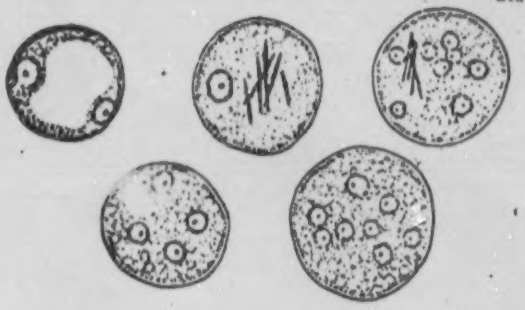


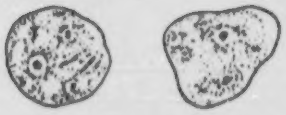
Entamoeba histolytica



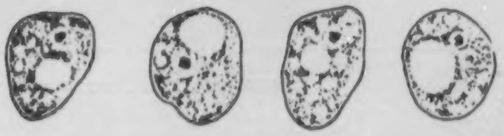


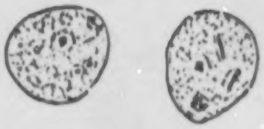
Entamoeba coli



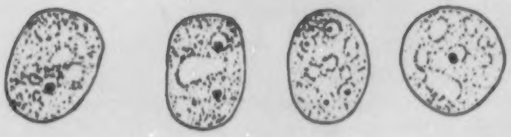


Iodamoeba butschlii





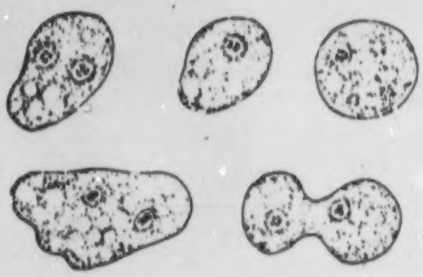
Endolimax nana



ANNEX

Identification Forms of Class Rhizopoda (continued)

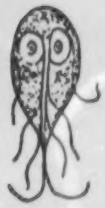
Trophozoites **Cysts**



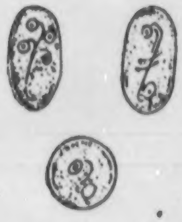
Dientamoeba fragilis

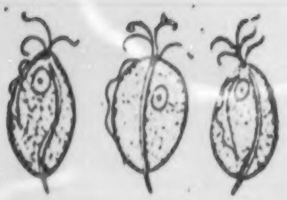
Identification Forms of Class Zoomastigophora

Trophozoites **Cysts**



Giardia lamblia





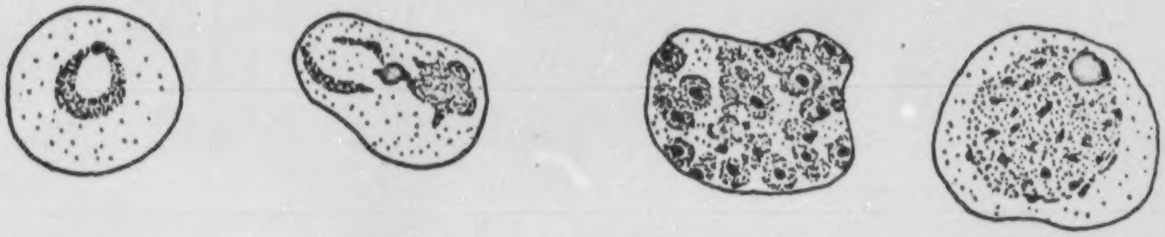
Trichomonas species

ANNEX

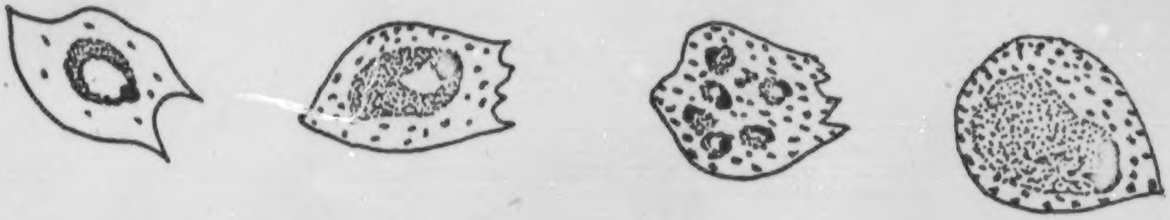
Identification Forms of Class Telesporea

Ring Form Trophozoites **Mature Trophozoites** **Schizonts** **Gametocytes**

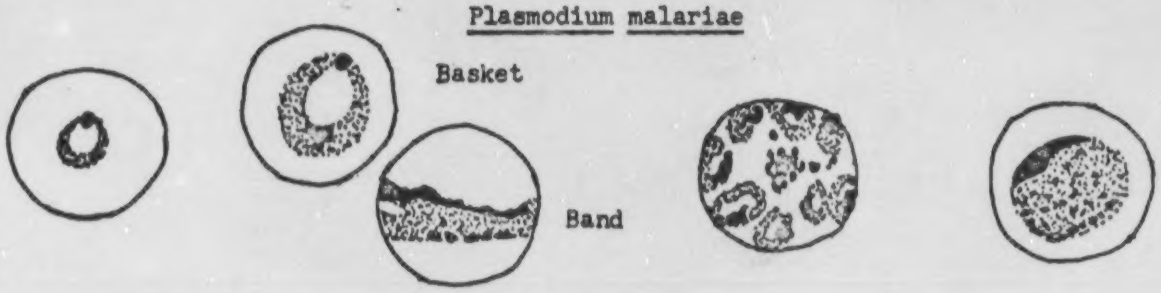
Plasmodium vivax



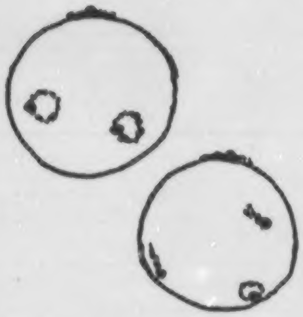
Plasmodium ovale

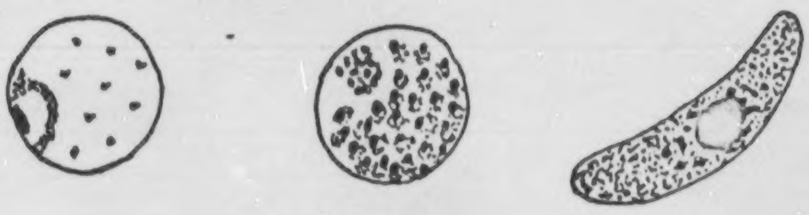


Plasmodium malariae



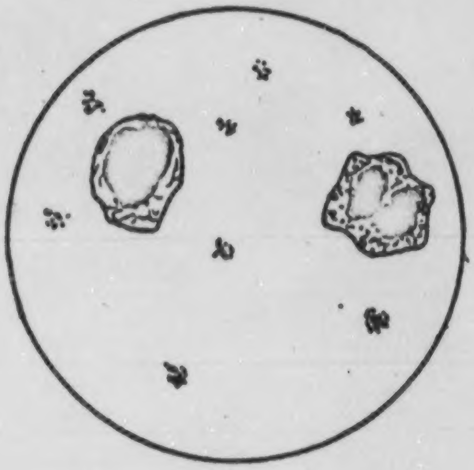
Plasmodium falciparum



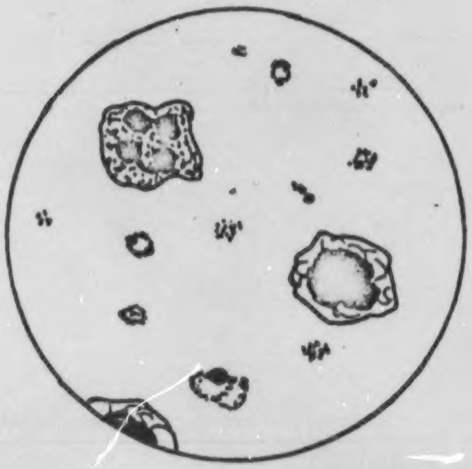


ANNEX

Identification Forms of Class Telosporea (continued)



Negative Thick Drop



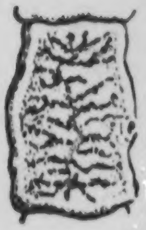
Positive Thick Drop

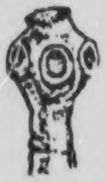
ANNEX

Identification Forms of Common Cestodes

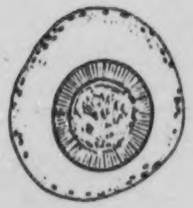
Proglottids Scolicies Eggs

Taenia solium

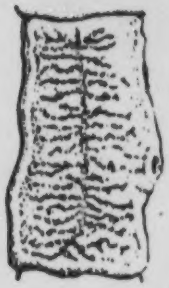


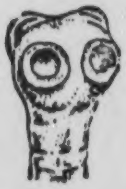


Taenia species

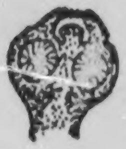


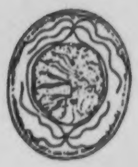
Taenia saginata



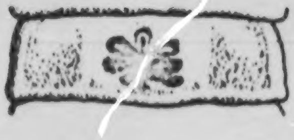


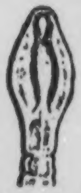
Hymenolepis nana

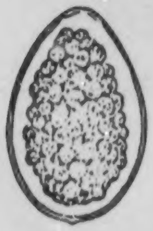




Diphyllobothrium latum



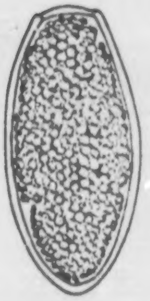


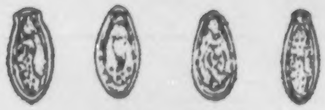


ANNEX

Identification Forms of Common Trematodes

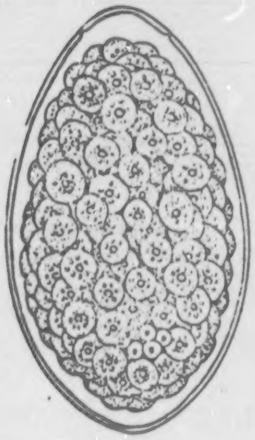
Paragonimus westermani



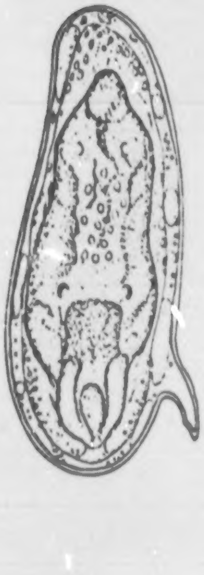


Clonorchis sinensis
and
related species

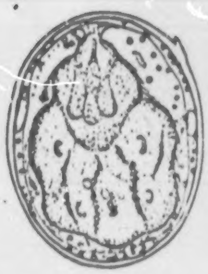
Fasciola hepatica



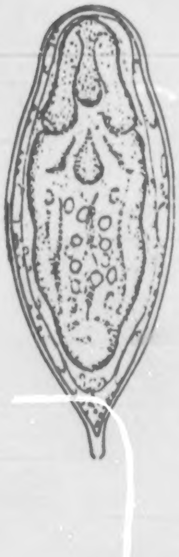
Schistosoma mansoni



Schistosoma japonicum

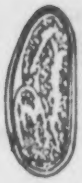


Schistosoma haematobium



ANNEX

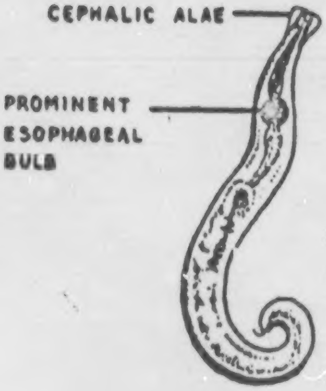
Identification Forms of Common Nematodes



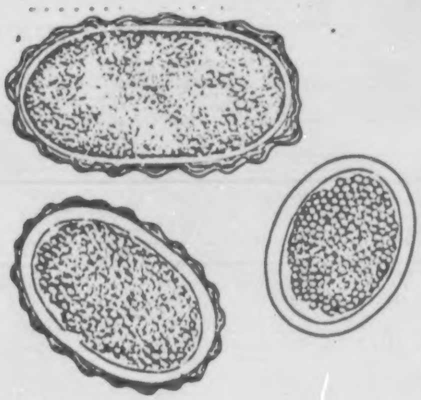
Enterobius vermicularis

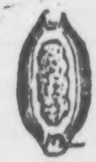
Egg

Adult


CEPHALIC ALAE
PROMINENT ESOPHAGEAL BULB

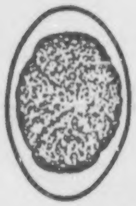
Ascaris lumbricoides Eggs





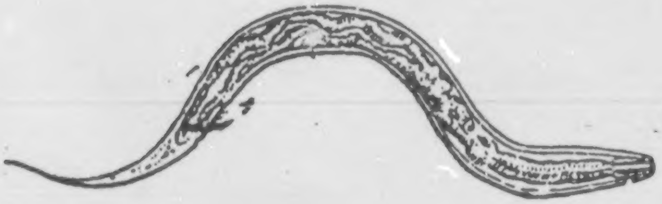
Trichuris trichiura

Egg

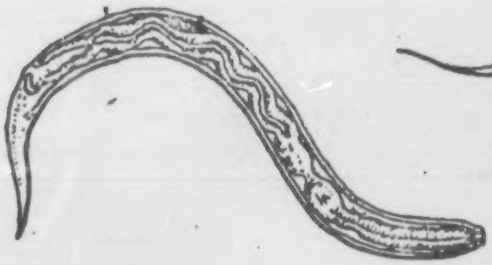


Hookworm species

Egg



Hookworm species
Rhabditiform larva



Strongyloides stercoralis
Rhabditiform larva

SSG G.B. Howe
LP 08-280-602
068

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

Parasitological Laboratory Techniques (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) course

PLACE: Classroom

REFERENCES: TM 8-227-2, pp 1-1 to 1-3, 5-1 to 5-4, 7-1 to 7-4

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601 -1

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, eraser, magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

LP 08-280-602-
068

I. INTRODUCTION (5 minutes)

A. Opening Statement: The identification forms of the medically important parasites may be found in a variety of specimens. Because of this, the laboratory technician must be prepared to properly process each type of clinical specimen; to apply certain staining techniques, and to recognize the colors these stains impart to parasites which may be present.

B. Objectives

1. Given a typical laboratory situation, select the proper storage and preservation procedure for a given parasitological specimen.

2. Select the correct staining reaction or purpose for a given stain or reagent.

3. Given a specific description or example, select or recognize the various colors and staining properties associated with the identification structures of the common intestinal protozoa.

4. Select the correct application of the stains or reagents taught in class.

C. Class Procedure and Lesson Tie-in

1. To be followed by a lecture on stool specimens.

2. To be followed by lectures on protozoans in which these staining properties are used for identification.

II. EXPLANATION (32 min)

A. Types of Specimens

1. Feces

a. Primary parasitological specimen

b. Many identification forms may be seen

NOTE: Discussed in detail in following lecture

2. Sputum- helminth eggs and larvae

NOTE: Refer students to GR 08-280-601, 068

3. Blood- malarial parasites and others (e.g. Trypanasoma sp.)

4. Tissue- protozoans and many helminthic forms

a. Lung

b. Brain

c. Muscle

5. Vaginal smears- protozoans

6. Scotch tape prep- Enterobius vermicularis eggs

B. Storage and Preservation of Specimens

1. Storage for short periods-

a. Refrigeration- 4-6°C up to 48 hours

b. Room temperature- no more than 30 minutes

NOTE: Trophozoites usually begin to deteriorate within 30-60 minutes

2. Storage and Preservation Indefinately

a. Merthiolate-Iodine-Formalin (M.I.F.)

(1) Forms preserved

(a) Trophozoites

(b) Cysts

(c) Eggs

(d) larva

(2) Advantage- easily concentrated

(3) Disadvantage- cannot be used for stained permanent mounts

b. Formalin

(1) Forms preserved

(a) cysts

(b) eggs

(c) larva

(2) Advantage- easily concentrated

(3) Disadvantage- cannot be used for stained permanent mounts

c. Polyvinyl Alcohol (PVA)

(1) Forms preserved

(a) Trophozoites

(b) Cysts

(2) Advantage- best for stained perminent mounts

(3) Disadvantage- Prolonged storage causes PVA to jell

d. Shipping of specimens

(1) Formalin best for helminth eggs and larva

(2) PVA best for protozoan cysts and trophozoites

C. Stains and Solutions

1. Temporary mounts

a. Warm physiological saline (0.85%)

(1) Permits observation of trophozoite motility

(2) Helminth eggs and larva may also be observed

(3) Does not stain organisms

b. Lugol's iodine

(1) Colors imparted by stain

(a) Cytoplasm - yellow

(b) Nucleii - black, refractile

(c) Glycogen - reddish brown

(d) Chromatiodal bar - siightly darker than cytoplasm

(e) Background - yellow

(2) Disadvantage - lyses trophozoites

c. Merthiolate-Iodine-Formalin

(1) Two phases

(a) Iodine phase - same as Lugol's Iodine

(b) Eosin phase - merthiolate

(2) Colors imparted by stain

(a) Cytoplasm - pink to red

(b) Nucleii - black, refractile

(c) Glycogen - colorless to light pink

(d) Chromatoidal bar - slightly darker than cytoplasm

(e) Background - pink

2. Permanent mounts

a. Iron Hematoxylin (Heidenhein's)

(1) Colors imparted by stain

(a) Cytoplasm - shades of grey

(b) Nuclear material and chromatiodal bar - black

(c) Glycogen - colorless

(d) Cyst wall - colorless

(e) RBCs and bacteria - black

(f) Background - grey

255

(2) Advantage - morphological details stain very distinctly

b. Trichrome (Gomori-Wheatley's)

(1) Colors imparted by stain

(a) Cytoplasm - blue-green to purple

(b) Nuclear material -deep red to black

(c) Chromatoidal bar- red

(d) Glycogen - colorless

(e) Eggs and larvae - red

(f) RBCs and bacteria - pink to red

(g) Background - green to light purple

(2) Advantage - quick and easy to perform

D. Questions From Students

III. SUMMARY (3 min)

A. Review of Main Points

1. Types of specimens

2. Storage and preservation

3. Stains and solutions

B. Closing Statement: By utilizing proper storage and preservation procedures, and by applying proper staining techniques, the laboratory technician can find identification forms of parasites which may be present in various types of specimens.

INSTRUCTIONAL HOURS

Number of instructors needed - - - 2

Primary hours	1
Secondary hours	0
TOTAL	1

1.

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-603
LABORATORY SCIENCES DIVISION 078
MICROBIIOLOGY BRANCH

Protozoan Organisms: Class Rhizopoda and Zoomastigophora (4 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp 1218-1219
Brown, H. W. Basic Clinical Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1969, pp 17-68
Cheng, T. C. General Parasitology, New York: Academic Press, Inc., 1973, pp 161-233

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601-1 issued to students at the beginning of the course

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; and magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

1. INTRODUCTION (5 min)

A. Opening Statement: The intestinal protozoans cause the largest incidence of parasitic infections of any group of parasites studied. They pose a major health problem throughout the world. Infections by some protozoans often result in irreparable damage and occasionally in death. For this reason, it is important that you, as laboratory technicians, be able to identify and report to the physician the various forms of the med-

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*This lesson plan supersedes LP 08-280-603, 017

ically important protozoans seen.

B. Objectives:

1. Given a list of the characteristics of the classes Rhizopoda and Zoomastigophora, select the correct characteristics for each of the classes.
2. Select from a list, the specimen of choice, mode of infection, and infective form of each of the protozoans studied in class.
3. Given a list of the identifying characteristics of the cystic and trophozoite stages of each of the protozoans studied in the class, identify these characteristics in a diagram of each organism and select the corresponding characteristics of each organism studied.
4. After practice, microscopically locate and identify from prepared slides, members of all classes studied.

C. Class Procedure and Lesson Tie-in

This instruction will be followed by demonstrations of the organisms presented and additional practical exercises

II. EXPLANATION (150 min)

A. Classification

1. Kingdom-Animal
2. Subkingdom-Protozoa
3. Phylum-Protozoa

B. Class Rhizopoda

1. General characteristics
    a. Geographic distribution-worldwide
    b. Habitat-large intestine
    c. Specimen-feces
    d. Infective form-mature cyst

e. Mode of infection-ingestion

f. Unicellular

g. Motility by means of pseudopoda

2. Entamoeba histolytica

a. General characteristics

(1) Pathogen

(2) Destroys tissue

(3) Causes amoebic dysentery

b. Identification forms

(1) Trophozoite

(a) Motility-rapid and directional

(b) Nucleus

[1] One round nucleus present

[2] Evenly distributed chromatin material around the nuclear membrane

[3] Pinpoint, centrally located karyosome

(c) Cytoplasm

[1] Finely granular

[2] Clean appearance

[3] RBC's mat be present

(2) Cyst

(a) Nucleus

[1] Morphologically identical to the trophozoite

[2] 1 to 4 nuclei present

            [3] Four nuclei in mature cyst

        (b) Cytoplasm

            [1] Shape-round

            [2] Cigar shaped, rounded end chromatoidal bars may be seen

            [3] Small, diffuse glycogen vacuole sometimes seen

3. Entamoeba coli

    a. General characteristics

        (1) Non-pathogen

        (2) May be confused with Entamoeba histolytica

    b. Identification forms

        (1) Trophozoite

            (a) Motility-sluggish and non-directional

            (b) Nucleus

                [1] One round nucleus present

                [2] Unevenly distributed chromatin material around the nuclear membrane

                [3] Karyosome eccentric and larger than Entamoeba histolytica

            (c) Cytoplasm

                [1] Coarsely granular

                [2] Dirty appearance

                [3] Vacuolated with bacterial inclusions

- (2) Cyst
  - (a) Nucleus
    - [1] Morphologically identical to trophozoite
    - [2] 1 to 8 nuclei present
    - [3] Eight nuclei in mature cyst
  - (b) Cytoplasm
    - [1] Shape-round
    - [2] Splintered end chromatoidal bars rarely seen
    - [3] Diffuse glycogen vacuole found in immature cyst *rarely* seen

4. *Endolimax nana*
   - a. General characteristics
     - (1) Non-pathogen
     - (2) May be confused with *Entamoeba histolytica*
   - b. Identification forms
     - (1) Trophozoite
       - (a) Motility-sluggish and non-directional
       - (b) Nucleus
         - [1] One present
         - [2] Large, irregular refractile karyosome
         - [3] Indistinct nuclear membrane
       - (c) Cytoplasm
         - [1] Granular

[2] Vacuolated with bacterial inclusions

(2) Cyst

(a) Nucleus

[1] Identical to trophozoite

[2] 1 to 4 refractile nuceli

[3] Four nuclei in mature cyst

(b) Cytoplasm

[1] Shape-round to oval

[2] Rare, diffuse glycogen vacuole seen

5. *Iodamoeba butschlii*

a. General characteristics

(1) Non-pathogen

(2) Often confused with *Entamoeba histolytica*

b. Identification forms

(1) Trophozoite

(a) Motility-sluggish and non-directional

(b) Nucleus

[1] One present

[2] Large, refractile karyosome

[3] Indistinct nuclear membrane

(c) Cytoplasm

[1] Coarsely granular

[2] Vacuolated with bacterial inclusions

(2) Cyst

(a) Nucleus

[1] Identical to trophozoite

[2] One present in mature cyst

(b) Cytoplasm

[1] Shape-irregular

[2] Large, distinct glycogen vacuole present

6. Dientamoeba fragilis

a. General characteristics

(1) Questional pathogen

(2) May cause persistent diarrhea

b. Identification form

(1) Trophozoite

(a) Motility-Active and directional

(b) Nucleus

[1] Usually two present (20% of the time one present)

[2] Fragmented karyosome

[3] Indistinct nuclear membrane

(c) Cytoplasm

[1] Finely granular

[2] Vacuolated with bacterial inclusions

(2) No cyst stage

LP 08-280-603
078

C. Class Zoomastigophora

1. General characteristics
    - a. Geographic distribution-worldwide
    - b. Unicellular
    - c. Motility by means of flagella
2. Giardia lamblia
    - a. General characteristics
        - (1) Pathogen
        - (2) Prevents absorption of fat and Vitamin A
        - (3) Habitat-small intestine
        - (4) Specimen-feces
        - (5) Infective form-mature cyst
        - (6) Mode of infection-ingestion
    - b. Identification forms
        - (1) Trophozoite
            - (a) Motility-resembles a "falling leaf"
            - (b) Nucleus-two present
            - (c) Axostyle
                - [1] Means of support
                - [2] Attachment of flagella
            - (d) Flageela-four pair (8) present
        - (2) Cyst
            - (a) Nucleus-two to four seen

(b) Cytoplasm

[1] Shape-oval

[2] Halo appearance around cytoplasm

[3] Contains remanants of axostyle and flagella

3. Trichomonas vaginalis

a. General characteristics

(1) Pathogen

(2) Habitat-vagina, urethra, prostrate gland

(3) Specimen-urine, urethral exudate

(4) Infective form-trophozoite

(5) Mode of infection-sexual contact

NOTE: Trichomonas vaginalis may be transmitted among females living under institutionalized conditions if proper sanitary practices are not carried out.

b. Identification form

(1) Trophozoite

(a) Motility-jerky and erratic

(b) Nucleus-one large nucleus present

(c) Shape-pyriform (pear)

(d) Flagella

[1] Anterior position

[2] 3 to 5 present

(e) Undulating membrane with attached flagella

(2) No cyst stage

      (3) Other species of *Trichomonas*-determined by specimen source
        (a) *Trichomonas tenax*-mouth
        (b) *Trichomonas hominis*-stool

D. Other Findings

- 1. Charcot-Leyden crystals
  - a. Indicative of intestinal bleeding
  - b. Appearance
    - (1) Elongate
    - (2) Double rhomboid shape
    - (3) Sharply pointed at both ends
  - c. Report if present
- 2. *Blastocystis hominis*
  - a. May be confused with protozoan cysts
  - b. Appearance
    - (1) Shape-oval to round
    - (2) Large central structureless mass
    - (3) Mass surrounded by refractile granules
- 3. Other yeast
  - a. May be confused with protozoan cysts
  - b. Appearance
    - (1) Shape-oval
    - (2) Demonstrate budding

4. Cellular elements from the intestinal tract
    - a. May be confused with protozoan trophozoites
    - b. Appearance
        - (1) Nucleus to cytoplasm ratio larger
        - (2) Nucleus more granular
        - (3) Cytoplasm more granular
    - c. Examples
        - (1) Epithelial cells
        - (2) Leukocytes

E. Question From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Classification
2. Class Rhizopoda
3. Class Zoomastigophora
4. Other findings

B. Closing Statement: You have been given the tools for the identification of the protozoans. With practice, you will be able to identify and report your findings to the physician. Your ability to perform your duties as a laboratory specialist will aid the physician in giving better care to his patients.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1

Primary hours	4
Secondary hours	0
TOTAL	4

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-604
LABORATORY SCIENCES DIVISON 068
MICROBIOLOGY BRANCH

Concentration Techniques (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCE: TM 8-227-2, pp 5-10 to 5-11; Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp1173-1174

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601, issued at the beginning of the course, 1 per student

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: VT 61; chalk, chalkboard, eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: Unless an individual with a parasitic infection is passing a large number of parasitic forms each day, your chances of recovering those forms are minimal without the use of a concentration technique. Two of the most commonly used methods will be presented to you.

B. Objective

Given examples of techniques used in processing of fecal specimens, correctly list the important steps used in performing the zinc sulfate centrifugal floatation technique.

C. Class Procedure and Lesson Tie-in

Students will perform the zinc sulfate centrifugal floatation technique at a later date

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*This lesson plan supersedes LP 08-280-604, 017

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II. EXPLANATION (35 min)

A. Formalin-Ether Concentration Technique

1. Advantages
    - a. Concentrates most organisms well
    - b. Good for specimens preserved in formalin
    - c. Easy to perform
2. Disadvantages
    - a. Storage of ether
    - b. Processing specimens using ether (volatile fluid)

NOTE: Show VT 61 (11:49 min)

B. Zinc Sulfate Centrifugal Floatation Technique

1. Advantages
    - a. Easy to perform
    - b. No volatile fluids used
2. Disadvantages
    - a. Not suitable for fatty stools
    - b. Does not float infertile *Ascaris* or *Trichuris* eggs or the eggs of most trematodes
3. Steps in the floatation technique
    - a. Place 1/3 to 1/2 ml. of feces in a separate container
    - b. Emulsify in 1 ml. of tap water
    - c. Add one 13x100 mm test tubefull of water and mix
    - d. Filter through 2 layers of wet gauze into a 15 ml. centrifuge tube

QUESTION: Why should the gauze be wet?

ANSWER: Eggs may stick to the dry gauze.

e. Centrifuge for one minute at 2500 rpm

f. Pour off supernatant fluid

g. Add one ml. of zinc sulfate solution (with a specific gravity of 1.18 to 1.20) and mix thoroughly

h. Fill the tube to the 13 or 14 ml. mark with zinc sulfate solution

i. Centrifuge for one minute at 2500 rpm

j. Carefully place the tube vertically in a rack without shaking or spilling

k. Gently slide a loop under the surface film and remove two or three loopfuls of the material to a glass microscope slide

l. Coverslip and examine

QUESTION: Where would you expect to find the parasitic forms in a zinc sulfate floatation technique?

ANSWER: The forms willbe in the surface film.

C. Question From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Formalin-Ether concentration technique

2. Zinc sulfate centrifugal floatation technique

B. Closing Statement: In order to successfully recover identification forms of parasites from a stool specimen, it will necessary for you to master these procedures. Your ability to do this will aid the physician in giving the patient rapid and effective treatment for parasitic infections.

SSG G.B. Howe
LP 08-280-~~[illegible]~~ 605
06[illegible]

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

The Stool Specimen (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

TM 8-277-2, pp 1-4, 2-1 to 4-5

Belding, D. L. Textbook of Parasitology, 3rd ed. New York: Appleton-Century-Crofts, Inc., pp 1170-1174.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Nome

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601—1

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, eraser and magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: Parasitic organisms are often present in human hosts long before they cause clinical symptoms. This is particularly true for the military services, since large numbers of troops often travel to and from areas where parasites are endemic. These troops may aquire parasitic organisms and unknowingly become carrier sources for transmission to others. Since identification forms of the majority of common parasites may be recovered from the feces, the laboratory technician must be familiar with examination procedures used for stool specimens.

B. Objective

. Given a list of procedures which may be performed on a stool specimen, select those which are routinely performed and be able to correctly report each procedure.

C. Class Procedure and Lesson Tie-in: None

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II. EXPLANATION (30 min)

- A. Macroscopic Examination
  - 1. Gross examination
    - a. Color - significance and report terminology
      - (1) Brown - normal
      - (2) Black
        - (a) may indicate intestinal bleeding (especially a black 'tarry' stool)
        - (b) May be caused by medication (Iron, Bismuth)
      - (3) Red
        - (a) May indicate intestinal bleeding
        - (b) May be caused by eating certain foods (tomatoes)
      - (4) Yellow
        - (a) May indicate unchanged bilirubin
        - (b) May be caused by milk consumption
      - (5) Green
        - (a) May indicate unchanged bilirubin
        - (b) May be caused by consumption of green vegátables
      - (6) Grey
        - (a) May be caused by excess fats or bile deficiecy
        - (b) May be caused by eating certain foods
    - b. Consistency
      - (1) Hard - resists puncture
      - (2) Rormed - maintains bowel shape when passed
      - (3) Soft - looses shape when passed
      - (4) Watery - pours easily (diarrheic)

NOTE: Protozoan trophozoites are most often recoverable from watery stools; usually not recoverable from hard or formed stools.

c. Mucous
  (1) appears as white patches on specimen
  (2) Report present or absent

NOTE: Patches of mucous, especially if bloody, may contain protozoan trophozoites.

d. Gross blood
  (1) blood visable on specimen
  (2) report present or absent

e. Occult blood
  (1) not visable
  (2) detected by chemical means (at physician's request)
    (a) Guiac method
    (b) Hemoccult method - most commonly used
  (3) report 1+, 2+, 3+, 4+, or NEG

B. Microscopic Examination

1. Direct examination

  a. Saline slide method
    (1) Primarily used to detect trophozoite motility
    (2) Perform within 60 minutes after collection
    (3) Reagent - warm physiological saline (0.85%)
    (4) Procedure
      (a) Place one drop of saline on slide

NOTE: Insure that slide and saline are warm; approximately 37°C (incubator temperature)

      (b) Mix in small amount of feces and coverslip the slide
      (c) Examine microscopically using hign dry objective

b. Iodine slide preparation

(1) Primarily used to detect protozoan cysts

(2) Trophozoites rapidly lysed

(3) Reagent - Lugol's Iodine

NOTE: Refer students to GR 08-280-601, 068 for preparation of Lugol's Iodine.

(4) Procedure

(a) Place one drop of Lugol's Iodine on slide

(b) Mix in a small amount of feces and coverslip the slide

(c) Examine microsopically using high dry objective

2. Fecal Fat - test performed upon physician's request

a. Sudan III stain used - fat globules stain red

b. Fat globules observed microscopically

c. Report number of fat globules/ high powered field

3. Concentration Techniques

a. Increases probability of recovering parasites

b. Methods

(1) Zinc Sulfate centrifugal floatation

(2) Formalin-ether concentration technique

NOTE: These methods will be discussed in detail in a subsiquent lecture

4. Reporting Microscopic Examination Findiggs

a. Negative - report "no ova or parasites seen"

NOTE: The term 'negative' is not used, since failure to observe ova or parasites in a single specimen or aliquot does not necessarily mean that the patient is free of parasites

b. Positive - report scientific name of parasite

C. Questions from Students

SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-606
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Identification of Organisms in All Classes Studied (31 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: All previous references

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Microscopes, lamps, prepared microscope slides of all organisms studied, immersion oil, lens paper, interval timer, 35 mm slide projector, screen, 35 mm photomicrographs of all organisms studied.

PERSONNEL: One instructor (MOS 92B3H) per six students

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical Exercise 1, Demonstration

I. INTRODUCTION (5 min)

A. Opening Statement: Organisms which are responsible for parasitic infections in man continue to thrive in many areas around the world. Because of the prevalence of these particular parasites, a great responsibility rests upon the laboratory specialist. No successful program for the treatment, control, or eradication of these parasitic diseases can be realized unless the diagnostic forms of these organisms are rapidly and accurately identified

B. Objective

Given prepared, mounted specimens containing identification forms of parasitic organisms studied, locate and identify the organisms present.

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* This lesson plan supersedes LP 08-280-606, 027

C. Class Procedure and Lesson Tie-in

1. Throughout the course, practice time has been allotted for students to locate and identify organisms on prepared slides.

II. APPLICATION (1225 min)

A. Demonstration, PE hours 1 and 2

1. Prior to class, microscope slides are set-up to display characteristic identification forms of the intestinal protozoa (see Annex A)

2. Prior to class, 35 mm photomicrographs of the intestinal protozoa are set-up for display to students. (see Annex A)

3. Arrangement of students

4. Directions to students

5. Students' activities

a. Microscope slides

(1) Move from microscope to microscope and study organisms displayed

(2) Utilize lecture notes for the identification characteristics of the protozoans

b. 35 mm photomicrographs- Review identification characteristics from the examples presented

6. Instructors' activites

a. Prior to class, set-up the demonstration practical exercise and the 35 mm photomicrographs to display characteristic identification forms of the intestinal protozoa (see Annex A)

b. Aid students in correctly identifying the identification forms of the intestinal protozoa.

B. Practice Scanning, PE hour 3

1. Prior to class, prepare slide study sets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activities

   a. Independently scan microscope slides from the slide study sets and determine what organisms are present on each slide.

   b. Scan only the M.I.F.'s and Giemsa stained slides

   c. Compare answers to answer key

5. Instructors' activites

   a. Prior to class, prepare slide study sets (see Annex C)

   b. Answer student questions

C. Demonstration, PE hours 4 and 5

1. Prior to class, microscope slides are set-up to display characteristic identification forms of the genus Plasmodium and the intestinal protozoa (see Annexes A and B)

2. Prior to class, 35 mm photomicrographs of the genus Plasmodium are set-up for display to students

3. Arrangement of students

4. Directions to students

   a. Microscope slides

      (1) Move from microscope to microscope and study the organisms displayed

      (2) Utilize lecture notes for the identification characteristics of the organisms displayed

   b. 35 mm photomicrographs - Review identification characteristics from the examples presented

5. Instructors' activites

   a. Prior to class, set-up the demonstration practical exercise and the 35 mm photomicrographs to display characteristic identification forms of the genus Plasmodium and the intestinal protozoa.

b. Aid students in correctly identifying the identification forms displayed

D. Practice Scanning, PE hours 6 and 7

1. Prior to class, prepare slide study sets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activites

   a. Independently scan microscope slides from the slide study sets for the presence of the intestinal protozoa

   b. Compare answers to answer key

5. Instructors' activities

   a. Prior to class, prepare slide study sets (see Annex C)

   b. Answer student questions

E. Practice Scanning, PE hour 8

1. Prior to class, prepare slide study sets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activities

   a. Independently scan microscope slides from the slide study sets for the presence of malarial parasites

   b. Compare answers to the answer key

5. Instructors' activities

   a. Prior to class, prepare slide study sets (see Annex C)

   b. Answer student questions

F. Demonstration, PE hour 9 - Practice Station to Station

1. Prior to class, microscope are set-up to display identifica-

tion forms of the classes Rhizopoda, Zoomastigophora, and the genus Plasmodium. (see Annexes A and B)

2. Arrangement of students

3. Directions to students

4. Students' activities

    a. Move from microscope to microscope at timed intervals

    b. Identify and record the organisms presented

5. Instructors' activites

    a. Prior to class, set-up the the demonstration practical exercise to display characteristic forms of the organisms (see Annexes A and B)

    b. Critique the practice station to station

G. Practice Scanning, PE hour 10

1. Prior to class, prepare study slide sets(see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activites

    a. Independently scan microscope slides from the slide sets for organisms studied in the classes Rhizopoda, Zoomastigophora, and the genus Plasmodium

    b. Compare answers to the answer key

5. Instructors' activities

    a. Prior to class, prepare slide study sets (see Annex C)

    b. Answer student questions

H. Demonstration, PE hours 11 and 12

1. Prior to class, microscope slides are set-up to display characteristic identification forms of the classes Cestoda,

Trematoda, and Nematoda (see Annex D)

2. Prior to class, 35 mm photomicrographs of the classes Cestoda, Trematoda, and Nematoda are set-up for display to students

3. Arrangement of students

4. Directions to students

   a. Microscope slides

      (1) Move from microscope to microscope and study the organisms displayed

      (2) Utilize lecture notes for the identification characteristics from the examples presented

   b. 35 mm photomicrographs - Review identification characteristics from the examples presented

5. Instructors' activities

   a. Prior to class, set-up the demonstration practical exercise and the 35 mm photomicrographs to display characteristic identification forms of the classes Cestoda, Trematoda, and Nematoda

   b. Aid students in correctly identifying the identification forms displayed

I. Practice Scanning, PE hours 13 and 14

1. Prior to class, prepare slide study dets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activites

   a. Independently scan microscope slides from the slide study sets for organisms in the classes Cestoda, Trematoda, and Nematoda

b. Answer student questions

J. Checklist Scanning, PE hours 15-18

1. Prior to class, prepare slide study sets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activities

   a. Use the prepared slide study set to locate and identify all organisms present

   b. Check the name of the organisms on the scan checklist (M 08-280-606-1)

   c. Verify the correctness of the results with an instructor

5. Instructors' activities

   a. Prior to class, prepare slide study sets (see Annex C)

   b. Verify and correct student results

K. Demonstration, PE hour 19 - Practice Station to Station

1. Prior to class, microscopes are set-up to display identification forms of the classes Rhizopoda, Zoomastigophora, Cestoda, Trematoda, and Nematoda and the genus Plasmodium (see Annexex A, B, and D)

2. Arrangement of students

3. Directions to students

4. Students' activities

   a. Move from microscope to microscope at timed intervals

   b. Identify and record the organisms presented

5. Instructors' activities

   a. Prior to class, set-up the demonstration practical exercise to display characteristic identification forms of the organisms presented (see Annexes A, B, and D)

   b. Critique the practice station to station

L. Practice Scanning, PE hours 20-31

1. Prior to class, prepare slide study sets (see Annex C)

2. Arrangement of students

3. Directions to students

4. Students' activities

   a. Using prepared slide study sets, independently locate and identify all organisms present

   b. Upon completion of all slides in the study set, have your answers verified by an instructor

5. Instructors' activities

   a. Prior to class, prepare slide study sets (see Annex C)

   b. Answer student questions

   c. Verify students answers

III. CRITIQUE (10 min)

A. Questions from students at the end of each block of instruction

B. Review identification characteristics of the organisms studied at the end of each block of instruction

C. Closing Statement: Repeated experience in identifying parasitic organisms is of great value in maintaining a high level of proficiency in the parasitology laboratory.

SSG Gerry Howe

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-607
LABORATORY SCINECES DIVISION 078
MICROBIOLOGY BRANCH

Malaria Organisms: Class Telosporea (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

Belding, D. L. Textbook of Parasitology, 3d Ed., New York; Appleton-Century-Crofts, Inc., 1965, pp 230-320
Brown, H. W. Basic Clinical Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1969, pp 43-44, 68-98
Cheng, T. C. General Parasitology, New York: Academic Press, Inc., 1973, pp 247-283
Garnham, P. C. C. Malarial Parasites and other Haemosporidia, Oxford: Blackwell Scientific Publications, 1966, pp 17-55

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601-1

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; and magnetic placards(see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: Malaria has been deeemed as one of the most vicious diseases of man, and rightly so. It has played a significant role in shaping history, to include the decline of the ancient Greek and Roman empires. Today, it still remains a major health problem in many parts of the world.

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*This lesson plan supersedes LP 08-280-607, 027

Since military personnel face the extraordinary risk of malarial infection due to assignments in highly endemic areas, it is necessary for the medical laboratory specialist to be able to identify the malarial parasites which infect man.

B. Objectives

1. From a list of charactersitics, select the characteristics which pertain to the class Telosporea.
2. Given a list of choices, select the infective forms, mode of infection, and intermediate host of organisms belonging to the genus Plasmodium.
3. Given a list of the various forms of Plasmodium species found in the erythrocytic cycle in man, select the correct developmental sequence.
4. From a list of examples, select the identifying criteria of each of the Plasmodium species.
5. From a given list, select the common name, fever-chill cycle, and geographic distribution of each of the species of the genus Plasmodium.
6. After practice, properly report whether given stained blood smears are positive or negative for Plasmodium species.

C. Class Procedure and Lesson Tie-in

This lecture will be followed by many hours of practical exerecise. using the microscope.

II. EXPLANATION (70 min)

A. Classification

1. Phylum-Protozoa
2. Class-Telosporea
3. Genus-Plasmodium
4. Species
    a. Plasmodium vivax

b. Plasmodium ovale

c. Plasmodium malariae

d. Plasmodium falciparum

B. General Characteristics of Class Telosporea

1. Intracellular parasites of blood and tissues

2. Sexual and asexual reproduction

3. Locomotion by body flexion or gliding

C. Hosts

1. Definitive (invertebrate) host-female Anopheles mosquito (sexual stage of life cycle)

QUESTION: What is the definition of a definitive host?

ANSWER: The host in which the sexual or adult stages in the life cycle of a parasitic organism occur.

2. Intermediate (vertebrate) host-man (asexual stage of life cycle called Schizogony)

D. Morphological Forms Occurring in Man

1. Sporozoite - Infective form to man

2. Trophozoite

a. Motile, feeding form within RBC or tissue cell

b. Nuclear chromatoidal dot surrounded by cytoplasm

NOTE: It is difficult to speciate young trophozoites of the human malarial species since they all begin their development in the RBC in the characteristic signet ring appearance.

NOTE: This process of asexual, intracellular reproduction of malarial parasites is called schizogony.

3. Schizont
   a. Stage of development within RBC or tissue cell where nuclear splitting occurs.
   b. Each unit (divided chromatin and small amount of cytoplasm) is called a merozoite.
   c. Each species contains characteristic number of merozoites.
4. Gametocytes
   a. Sexually differentiated malarial parasite within RBC
      (1) Macrogametocyte-female
      (2) Microgametocyte-male
   b. Infective forms to mosquito

NOTE: The gametocytes of the human plasmodia are difficult to speciate except for Plasmodium falciparum.

SECOND HOUR

E. Characteristics of Specific Plasmodium species

NOTE: This portion may be presented in the form of a chart (see Annex C). To facilitate comparisons and contrasts among the species, use chalkboard drawings as appropriate (see Annex C). Malarial parasites are stained with the Romanovsky type stains. Therefore, all descriptions refer primarily to Giemsa stained slides where all nuclear chromatin stains reddish and all cytoplasm stains bluish.

1. Plasmodium vivax
   a. Common name-benign tertian
   b. Geographic distribution
      (1) Temperate zones
      (2) Tropics and subtropics

c. Fever-chill cycle: 45 hours

d. Appearance of infected RBC

  (1) Pale, enlarged

  (2) Fine Schnuffner's granulation-pinkish dots, like

[illegible] grains of sand

(3) Shape may be bizarre especially as organism matures

e. Trophozoite

(1) Large chromatin dot

(2) Amoeboid, difused appearing, vacuolated cytoplasm

f. Schizont

(1) 12-24 merozoites

(2) Parasite usually fills RBC

(3) Hemozoin pigment-yellowish brown

g. Gametocyte

(1) Parasite usually fills RBC

(2) Shape-circular to oval

(3) Abundant yellow-brown hemozoin pigment

NOTE: You are more likely to see all three morphological stages in one blood film with Plasmodium vivax than with other species.

2. Plasmodium ovale

a. Common name-ovale malaria

b. Geographic distribution-Central Africa

c. Fever-chill cycle: 49 hours

d. Appearance of infected RBC

(1) Enlarged, but less than Plasmodium vivax

(2) Numerous coarse, irregular, Schuffner's granuales-reddish

NOTE: Schuffner's granules in Plasmodium ovale tend to be slightly more concentrated toward the periphery of the RBC.

(3) Oval and fimbriated shape often seen

(4) Usually extremely pale

e. Trophozoite

(1) Large chromatin dot

(2) Compact, deep staining cytoplasm-very little vacuolation

f. Schizont

(1) 4-16 merozoites (average-8)

(2) Merozoites often larger than those of other species

(3) Hemozoin pigment-yellowish brown

g. Gametocyte

(1) Parasite rarely fills RBC

(2) Somewhat smaller than gametocytes of Plasmodium vivax

(3) Abundant dark brown hemazoin pigment

3. Plasmodium malariae

a. Common name-quartan malaria

b. Geographic distribution-tropics and subtropics

c. Fever-chill cycle: 72 hours

d. Appearance of infected RBC

(1) Normal shape, size, and color

(2) Usually no granulation seen

NOTE: Pinkish dustlike granules called Ziemanns' dots are rarely demonstrated.

e. Trophozoite

(1) Large chromatin dot

(2) Compact, dense cytoplasm-very little vacuolation observed

(3) Band forms

(4) Basket forms

f. Schizont

(1) 6-12 merozoites

(2) Often arranged in rosette pattern

(3) Central cluster of greenish, granular, hemozoin pigment

NOTE: Plasmodium malariae tends to produce more abundant amounts of hemozoin pigment

g. Gametocyte

(1) Parasite fills RBC

(2) Abundant dark green to black hemozoin pigment

4. Plasmodium falciparum

a. Common name-malignant tertian

b. Geographic distribution-tropics and subtropics

NOTE: Plasmodium falciparum is responsible for the highest incidence of malaria in the tropics and subtropics and also causes the most serious disease manifestations.

c. Fever-chill cycle: 36-48 hours

d. Appearance of infected RBC

(1) Normal size, shape,and color

NOTE: _______ blood smears, RBC's may have a copper color.

(2) Usually no granulation

NOTE: _____ _____ clefts may occasionally be seen especially _____ of the stain used is above 7.4 They may appear as _____ or angular red spots.

e. Trophozoite

(1) Small chromatin dot

(2) Delicate ring of cytoplasm

(3) Applique form

(4) Double ring form

(5) Multiple infection

NOTE: _____ infected RBC's are much more common with Plasmodium malariae than with other species.

f. Schizont

(1) 8-32 merozoites

(2) Black clumped hemozoin pigment

(3) Very rarely seen

g. Gametocyte

(1) Cresent shaped

(2) RBC may be ruptured

(3) More commonly seen than the gametocytes of other species

5. Questions from students

REVIEW (5 min.)

a. Review of main points

SSG G. B. Howe

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-608
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Giemsa Stain (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: TM 8-227, pp 11-1 to 11-5, GR 08-28--601-1.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: GR o8-280-601-1 issued at the beginning of the course, 1 per student

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: VT 08-280-608; chalk, chalkboard, eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: In order to identify and speciate malarial parasites, it is necessary that the staining characteristics which reveal the specific morphology of each organism be readily seen. This can only be accomplished when the laboratory technician employs the most effective techniques in preparing and staining blood smears. The Giemsa method is most commonly used for this purpose.

B. Objectives

1. Correctly explain the purpose of the reagents used in the Giemsa staining technique.

2. Given the steps in preparing and staining thick and thin blood smears for malaria identification using Giemsa stain, select the proper sequence of events.

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*This lesson plan supersedes LP 08-280-608, 027

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C. Class Procedure and Lesson Tie-in

The Giemsa staining technique is the most common used to identify malarial parasites. All the malarial slides used in this course were prepared in this manner.

II. EXPLANATION (30 min)

A. Points to be Observed in VT 08-280-608

1. Performance of capillary puncture

2. Application of blood to slide

3. Preparation of thin and thick smears

4. Air drying of slide

5. Reagents and their functions

6. Staining procedure

B. Show VT 08-280-608 (13 min)

C. Questions from Students About VT 08-280-608

QUESTION: What is the action of a mordant?

ANSWER: To prepare the cells to receive the stain

D. Microscopic Examination

1. Colors imparted by stain

a. Blood components - same as Wright's stain

b. Malarial parasites

(1) Chromatin - reddish to magenta

(2) Cytoplasm - smokey blue

(3) Hemazoin pigment - yellow or green to black, refractile

c. Stain precipitate - amorphous, easily refractile, extracellular

NOTE: Stress that malarial parasites appear as combinations of condensed red chromatin and blue cytoplasm, while platelets are purplish in color.

2. Scan slide on oil immersion

   a. Thick smear - to detect presence of malarial parasites

QUESTION: Will parasites be visible within RBC's in the thick drop?

ANSWER: No, RBC's will have been dehemaglobinized and will not be visible.

(1) If malarial parasites are present, they will appear as small red chromatin dots surrounded by a small amount of bluish cytoplasm

(2) If malarial parasites are seen or if doubt exists after scanning at least 50 oil immersion fields, scan the thin smear to speciate and/or confirm

(3) Do not attempt to speciate from the thick smear unless the gametocytes of Plasmodium falciparum are seen

   b. Thin smear - scan on oil immersion

(1) Scan feathered edge to speciate the genus Plasmodium or to confirm doubtful thick smear results

(2) Scan by overlapping fields until certain of species

(3) If in doubt about thick smear result, scan at least 10 minutes

E. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. VT 08-280-608

   a. Capillary puncture

   b. Preparation of thick and thin smears

   c. Reagents and their functions

   d. Staining procedure

2. Microscopic examination

B. Closing Statement: Giemsa stain is the most commonly used stain for malarial parasites. To facilitate rapid and accurate identification of malarial parasites, the laboratory technician must be proficient with this important technique.

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-611
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

The Class Cestoda (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp 564-644
Brown, H. W. Basic Clinical Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1969, pp 169-205
Cheng, T. C. General Parasitology, New York: Academic Press, Inc., 1973, pp 474-541

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, and magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: Morphologically the tapeworms are the simplest helminths known to man. Their simple body structure is designed to allow the host to perform complex matabolic processes. If you can imagine a worm measuring up to fifty feet lodged in your intestinal tract, you will begin to understand the damage it could do while competing with you for your food. Their ability to absorb vital nutrients and to cause chronic pathological processes necessitates their recovery and identification by the laboratory specialist.

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*This lesson plan supersedes LP 08-280-611, 017

B. Objectives:

1. Given a list of characteristics, select those characteristics that belong to the Cestoda.

2. Select from a list the common name, geographic distribution, habitat, specimen of choice, intermediate hosts, infective form, mode of infection, and identification forms of organisms belonging to the class Cestoda.

3. Given a list of identifying characteristics of the cestodes studied, math these characteristics to diagrams of the organisms.

4. After practice, microscopically locate and identify from prepared slides, members of the class Cestoda studied in class.

C. Class Procedure and Lesson Tie-in

One hour of demonstration followed by three hours of practical exercise on all helmiths studied

II. EXPLANATION (70 min)

A. Classification

1. Kingdom - Animal

2. Subkingdom - Metazoa

3. Phylum - Platyhelminthes

4. Class - Cestoda

B. General Characteristics

1. Flat (dorso-ventrally)

2. Segmented

3. Monecieous

4. No digestive tract

5. Adult composed of three regions

a. Scolex - location for organs of attachment

- (1) Spherical with four sucking discs; armed or unarmed
- (2) Almond, or spoon shaped with two sucking grooves

b. Neck - germinative portion of worm

c. Strobila - composed of proglottids (segments)

- (1) Immature proglottids
- (2) Mature proglottids
- (3) Gravid proglottids

C. Order Cyclophyllidea

1. Taenia solium

a. Common name-pork tapeworm

b. Geographic distribution-areas of the world where pork is raised for food

c. Habitat-small intestine

d. Specimen-feces

e. Infective form-cysticercus larva

f. Mode of infection-ingestion

g. Intermediate host

- (1) Pig
- (2) Man-accidental intermediate host
    - (a) Man ingests egg
    - (b) Causes cysticercosis

h. Identification forms

- (1) Egg
    - (a) Spherical

    - (b) Brown in color
    - (c) Radially striated embryophore
    - (d) Contains a hexacanth oncosphere
    - (e) Reported only as Taenia species
  - (2) Gravid proglottid
    - (a) Longer than wide
    - (b) seven to thirteen lateral uterine branches
  - (3) Scolex-armed rostellum

2. Taenia saginata
  - a. Common name-beef tapeworm
  - b. Geographic distribution-areas of the world where beef is raised for food
  - c. Habitat-small intestine
  - d. Specimen-feces
  - e. Infective form-cysticercus larva
  - f. Mode of infection-ingestion
  - g. Intermediate host-cow
  - h. Identification forms
    - (1) Egg-same as Taenia solium
    - (2) Gravid proglottid
      - (a) Longer than wide
      - (b) Fifteen to thirty lateral uterine branches
    - (3) Scolex-unarmed

3. Hymenolepsis nana
   - a. Common name-dwarf tapeworm
   - b. Geographic distribution-worldwide
   - c. Habitat-small intestine
   - d. Specimen-feces
   - e. Infective form-mature egg
   - f. Mode of Infection-ingestion
   - g. Autoinfective
   - h. Identification forms
     - (1) Egg
       - (a) Round to oval
       - (b) Hyaline color
       - (c) Granulated embryophore
       - (d) Contains a hexacanth oncosphere
       - (e) Bipolar filaments
     - (2) Gravid proglottid-rarely seen
     - (3) Scolex
       - (a) Retractable rostellum
       - (b) Armed

QUESTION: What cestode is autoinfective?

ANSWER: Hymenolepsis nana.

D. Order Pseudophyllidea-Diphyllobothrium latum

1. Common name-broadfish tapeworm

2. Geographic distribution-lake regions of the world
3. Habitat-small intestine
4. Specimen-feces
5. Infective form-pleurocercoid larva
6. Mode of infection-ingestion
7. Intermediate hosts
    a. First-copepod
    b. Second-fresh water fish
8. Identification forms
    a. Egg
        (1) Hen's egg shape
        (2) Hyaline to light yellow color
        (3) Operculated at small end
        (4) Abopercular button
        (5) Slightly thickened, single shell wall
        (6) Contains a yolk mass
    b. Gravid proglottid
        (1) Wider than long
        (2) Rosette shaped uterus
    c. Scolex
        (1) Almond, or spoon shaped
        (2) Pair of sucking grooves

__QUESTION:__ What is the second intermediate host of _Diphyllobothrium latum_?

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-612
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

The Class Trematoda (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

TM 8-227-2, Aug 1974

Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp 645-795
Brown, H. W. Basic Clinical Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1969, pp 206-250
Cheng, T. C. General Parasitology, New York: Academic Press, Inc., 1973, pp 377-457

STUDY ASSIGNMENT: After class study GR 08-280-601-1

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601-1 issued to students at the beginning of the course

PERSONNEL: One Instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: One of the most interesting classes of organisms studied in the field of parasitology is the class Trematoda. Due to the increased world travel and the assignment of military personnel in endemic areas, the importance of trematodes has increased in recent years. As medical laboratory specialists, you will be required to identify the medically important trematodes in order to enable the physician to treat the patient as soon as possible.

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*This lesson plan supersedes LP 08-280-612, 017

B. Objectives:

1. Given a list of characteristics of parasitic organisms, select the characteristics that belong to the class Trematoda.

2. Select from a list of examples the common name, geographic distribution, habitat, specimen of choice, intermediate hosts, infective form, mode of infection, and identification forms of organisms belonging to the class Trematoda.

3. Given a list of identifying characteristics of the trematodes studied in class, match these characteristics to diagrams of the organisms which illustrate each structure.

4. After practice, microscopically locate and identify from prepared slides, identifcation forms of the trematodes studied in class.

C. Class Procedure and Lesson Tie-in

One hour of demonstration followed by three hours of practical exercise on all helminths studied

II. EXPLANATION (70 min)

A. Classification

1. Kingdom-Animal

2. Subkingdom-Metazoa

3. Phylum-Platyhelminthes

4. Class-Trematoda

B. General Characteristics

1. Flat (leaf-like)

2. Unsegmented

3. Possess two suckers

- a. Oral
- b. Ventral (acetabulum)

4. Incomplete digestive system

C. Monecious Trematodes

1. General characteristics
    - a. Acquired by ingestion
    - b. Two intermediate hosts
        - (1) First is always a fresh water snail
        - (2) Second varies with each parasite
    - c. Infective form to man-metacercaria
    - d. All eggs operculated
2. Paragonimus westermani
    - a. Common name-Oriental lung fluke
    - b. Geographic distribution-Orient
    - c. Habitat-lungs
    - d. Specimen-sputum (40% recovery in feces)
    - e. Intermediate hosts
        - (1) First-snail
        - (2) Second-fresh water crab or crayfish
    - f. Identification form-Egg
        - (1) Ovoid or vase shaped
        - (2) Golden brown color
        - (3) Flattened operculum at broad end
        - (4) Abopercular thickening

        (5) Yolk mass fills egg

3. Clonorchis sinensis

    a. Common name-Chinese liver fluke

    b. Geographic distribution-Orient

    c. Habitat-biliary passages of the liver

    d. Specimen-Feces

    e. Intermediate hosts

        (1) First-snail

        (2) Second-fresh water fish

    f. Identification form-Egg

        (1) Ovoid or vase shaped

        (2) Yellow brown color

        (3) Operculum with prominent shoulders

        (4) Abopercular comma

        (5) Miracidium inside

        (6) Similiar eggs

            (a) Opisthorchis species

            (b) Metagonimus yokogawai

            (c) Heterophyes heterophyes

4. Fasciola hepatica

    a. Common name-Sheep liver fluke

    b. Geographic distribution-Worldwide in sheep-raising areas

    c. Habitat-liver

- d. Specimen-feces
- e. Intermediate hosts
  - (1) First-snail
  - (2) Second-aquatic plants
- f. Reservoir host-Sheep
- g. Identification form-Egg
  - (1) Ovoid shape
  - (2) Yellowish-brown color
  - (3) Indistinct operculum
  - (4) Yolk mass fills egg
  - (5) Similiar egg-_Fasciolopsis buski_

QUESTION: What developmental stage of the monecious trematodes is the infective form to man?

ANSWER: Metacercaria

- D. Diecious Trematodes
  - 1. General characteristics
    - a. Acquired by active penetration
    - b. One intermeduate host-fresh water snail
    - c. Infective form to man-cercaria
    - d. Habitat-venules (blood flukes)
    - e. Non-operculated eggs with a spine and miracidium inside
  - 2. _Schistosoma mansoni_
    - a. Common name-Manson's blood fluke
    - b. Geographic distribution-Africa, Latin America, Puerto Rico

- c. Habitat-venules of the large intestine
- d. Specimen-feces
- e. Identification form-Egg
  - (1) Elongate shape
  - (2) Yellowish-brown color
  - (3) Conspicuous lateral spine
  - (4) Miracidium inside

3. Schistosoma japonicum
   - a. Common name-Oriental blood fluke
   - b. Geographic distribution-Orient
   - c. Habitat-venules of the small intestine
   - d. Specimen-feces
   - e. Identification form-Egg
     - (1) Spherical to ovoid shape
     - (2) Yellowish-brown color
     - (3) Small, inconspicuous spine
     - (4) Leaf-like miracidium inside
     - (5) Sticky surface with fecal debris attached
4. Schistosoma haematobium
   - a. Common name-Vesical blood fluke
   - b. Geographic distribution-Africa
   - c. Habitat-venules of the urinary bladder
   - d. Specimen-urine (occasionally feces)

e. Identification form-Egg

(1) Elongate shape

(2) Hyaline color

(3) Conspicuous terminal spine

(4) Miracidium inside

QUESTION: What is the first intermediate host of all trematodes that infect man?

ANSWER: A fresh water snail.

E. Question From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Classification

2. General characteristics

3. Monecious trematodes

4. Diecious trematodes

B. Closing Statement: With practice obtained during the practical exercises, you will be able to identify the trematodes encountered in the medical laboratory.

INSTRUCTIONAL HOURS

Number of instructor required - - - 1

Primary hours	2
Secondary hours	0
TOTAL	2

MODIFICATIONS

LP-08-280-613 of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material was not available at time of publication and its exclusion does not alter the value of remaining material.

1

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-614
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

The Class Nematoda (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

Belding, D. L. Textbook of Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1965, pp 346-558
Brown, H. W. Basic Clinical Parasitology, 3d Ed., New York: Appleton-Century-Crofts, Inc., 1969, pp 99-168
Cheng, T. C. General Parasitology, New York: Academic Press, Inc., 1973, pp 586-689

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: GR 08-280-601-1

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, and magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Conference

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: Organsims belonging to the Nematoda are responsible for the highest worldwide incidence of human parasitosis caused by helminths. The majority of nematode infections occur in warm climates where sanitary practices are poorly developed. Therefor, it is important that laboratory specialists, stationed in endemic areas, be able to recognize diagnostic

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*This lesson plan supersedes LP [illegible]0-614, 017 diagnostic forms in specimens taken from suspected cases so that effective treatment can be initiated.

B. Objectives

1. Given a list of characteristics of parasitic organisms, select those characteristics that belong to the class Nematoda.

2. Select from a list of examples the common name, geographic distribution, habitat, specimen of choice, infective form, mode of infection, and identification forms of the organisms belonging to the class Nematoda.

3. Given a list of the identifying characteristics of the nematodes studied in class, match these characteristics to diagrams of the organsisms which illustrate each structure listed.

4. After practice, microscopically locate and identify from prepared slides, identification forms of the nematodes studied in class.

C. Class Procedure and Lesson Tie-in

1. The last two hours of lectures on the helminths

2. Later hours devoted to viewing parasites under the microscope

II. EXPLANATON (70 min)

A. Classification

1. Kingdom-Animal

2. Subkingdom-Metazoa

3. Phylum-Aschelminthes

4. Class-Nematoda

B. General Characteristics

1. Round (cylindrical)

2. Diecious (separate sexes)

2. a. Male smaller than female

   b. Male has curved tail

3. Complete digestive system

C. Nematodes Acquired by Ingestion of Infective Form

1. Enterobius vermicularis

   a. Common name-pinworm, seatworm

   b. Geographic distribution-worldwide

   c. Habitat-large intestine

   d. Specimen-scotch tape prep

NOTE: The best time to collect a pinworm specimen is after long periods of rest due to the migration of the female to the perianal foolds to deposit her eggs.

   e. Infective form-mature egg

   f. Autoinfective (eggs mature in 4-6 hours)

   g. Identification forms

      (1) Egg

         (a) Flattened on one side

         (b) Hyaline color

         (c) Triple-layered shell wall

         (d) Larva present in egg

            [1] Tadpole form

            [2] Vermiform larva

      (2) Adult

         (a) Cephalic alae

         (b) Esophogeal bulb

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QUESTION: What is the method of choice for recovering eggs of *Enterobius vermicularis*?

ANSWER: Scotch tape prep

2. *Ascaris lumbricoides*
    - a. Common name-giant intestinal roundworm
    - b. Geographic distribution-worldwide
    - c. Habitat-small intestine
    - d. Specimen-feces
    - e. Infective form-mature egg
    - f. Identification form-Egg
        - (1) Thick shell wall
        - (2) Corticated, fertilized
            - (a) Round to oval
            - (b) Brown color
            - (c) Yolk mass withdrawn from shell wall
            - (d) Bumpy appearing shell wall
        - (3) Decorticated, fertilized
            - (a) Round to oval
            - (b) Hyaline to yellow color
            - (c) Yolk mass withdrawn from shell wall
            - (d) Smooth shell wall
        - (4) Corticated, unfertilized
            - (a) Oval, elongate
            - (b) Brown color

(c) Yolk mass fills egg

(d) Bumpy appearing shell wall

(5) Decorticated, unfertilized-rarely seen

QUESTION: What is the common name of *Ascaris lumbriocides*?

ANSWER: The giant intestinal roundworm.

3. *Trichuris trichiura*
   - a. Common name- whipworm
   - b. Geographic distribution-worldwide
   - c. Habitat-large intestine
   - d. Specimen-feces
   - e. Infective form-mature egg
   - f. Identification forms
     - (1) Egg
       - (a) Football shaped
       - (b) Brown shell
       - (c) Bipolar mucoid plugs
       - (d) Yolk mass inside
     - (2) Adult-resembles a bullwhip

D. Nematodes Acquired by Active Penetration of the Infective Form

1. Hookworm species (*Ancylostoma duodenale* and *Necator americanus*)
   - a. Common name-old world hookworm and new world hookworm
   - b. Geographic distribution-worldwide in warm areas

c. Habitat-small intestine

d. Specimen-feces

e. Infective form-filariform larva

f. Identification forms

(1) Egg

(a) Bluntly rounded ends

(b) Hyaline color

(c) Single, thin shell wall

(d) Cleaved yolk mass

(2) Rhabditiform larva

(a) Long buccal cavity

(b) Small, inconspicuous genital primordium

(c) Long tapered tail

QUESTION: What is the habitat of the human hookworms?

ANSWER: The small intestine.

2. Strongyloides stercoralis

a. Common name-threadworm

b. Geographic distribution-worldwide in warm areas

c. Habitat-small intestine

d. Specimen-feces

e. Infective form-filariform larva

f. Autoinfective

NOTE: May be autoinfective if the rhabditiform larva hatches while in the intestine and develops to a filariform larva. The filariform larva can penetrate the intestine and repeat the life cycle.

g. Identification forms

(1) Egg-rarely seen

(2) Rhabditiform larva

(a) Short buccal cavity

(b) Large, conspicuous genital primordium

(c) Short, stubby tail

NOTE: The tail is not very well differentiated between hookworm species and Strongyloides stercoralis and is the poorest of the three criteria to use for identification.

E. Question From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Classification
2. General characteristics
3. Nematodes acquired by ingestion of infective form
4. Nematodes acquired by active penetration of infective form

B. Closing Statement: Correct identification of the parasitic nematodes is of utmost importance to the medical laboratory technician. Effective definitive care for the patient can only be accomplished after proper identification has been made.

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MODIFICATIONS

LP 08-280-609; 08-280-610 of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material was not available at time of publication and its exclusion does not alter the value of remaining material.

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-615
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Zinc Sulfate Centrifugal Floatation Practical Exercise (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: TM 8-227-2, pp 5-10 to 5-11

STUDY ASSIGNMENT: Before class study GR 08-280-601

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: Clean microscope slides; coverslips; Lugol's iodine; applicator sticks; saline; zinc sulfate solution with a specific gravity of 1.118 to 1.20; bacteriological loop; 15 ml. graduated centrifuge tubes; stool containers; lids; tongue depressors; funnels; gauze 4x4 (students should bring their own stool samples to class)

PERSONNEL: One instructor (MOS 92B3H) per six students

INSTRUCTIONAL AIDS: Chalk, chalkboard and eraser; microscopes, lamps; centrifuges

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical Exercise 1

FIRST AND SECOND HOURS

I. INTRODUCTION (5 min)

A. Opening Statement: The ability of the physician to accurately diagnose parasitic infections is often related to the laboratory technician's proficiency at demonstrating diagnostic forms in submitted specimens. Recovering and detecting significant parasitic forms in a stool specimen is greatly enhanced by the use of the zinc sulfate centrifugal floatation technique. During this exercise we will perform those steps whcih are important for effectively concentrating the fecal specimen.

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*This lesson plan supersedes LP 08-280-615, 017

B. Objective

Given the necessary materials, correctly perform the zinc sulfate centrifugal floatation technique.

C. Class Procedure and Lesson Tie-in

To familiarize the student with a technique for the concentration of parasitic organisms

II. APPLICATION (70 min)

A. Instructors' Activities

1. Write the steps in the zinc sulfate centrifugal floatation technique on the chalkboard as per GR 08-280-601
2. Supervise student activities
3. Assist students when required and correct mistakes

B. Students' Activities

1. Perform a macroscopic examination on their feces
2. Perform a direct microscopic examination on their feces
3. Concentrate their feces by the zinc sulfate centrifugal floatation technique as per GR 08-280-601
4. Perform microscopic examination of their concentrated feces
5. Clean up as directed

III. REVIEW OR CRITIQUE (5 min)

A. Questions From Students

B. Review of Main Points

1. Macroscopic examination
2. Direct microscopic examination
3. Concentration method

SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-616
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Station to Station (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: Scan 11 previous references

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Microscopes and microscope lamps

PERSONNEL: One primary instructor (MOS 92B3H) per 6 students

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, and prepared, keyed slides

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical Exercise 1

I. INTRODUCTION (5 min)

A. Opening Statement: The purpose of this examination is to evaluate your progress made in the study of clinical parasitology. Your accomplishment will reflect not only how well you have learned the identifying features of the parasitic organisms, but also your ability to apply this knowledge to actual parasite identification.

B. Objective

Given a specific microscopic field of vision at a series of microscopic stations, apply the proper identification criteria given in class and identify the organisms present.

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* This lesson plan supersedes LP 08-280-616, 068.

C. Class Procedure and Lesson Tie-in

1. Conduct as a practical examination

2. Laboratory tables equipped with microscopes and lamps

3. Students identify organisms displayed at each station and move in sequence at 45 second intervals from microscope to microscope

II. APPLICATION (30 min)

A. Prior to class, micrscopes are set-up and numbered in sequence to display representative identification forms of the classes Rhizopoda, Zoomastigophora, Cestoda, Trematoda, Nematoda, and the genus Plasmodium. (see Annex A)

B. Arrangement of Students

C. Directions to Students

D. Students' Activities

1. Move from microscope to microscope at timed intervals

2. Identify and record the organisms displayed

3. Turn in results to the instructor

E. Instructors' Activities

1. Prior to class set-up the demonstration practical exercise in numbered sequence to display representative identification forms of the organisms (see Annex A)

2. Regulate student movements from microscope to microscope at 45 second intervals

3. Collect and grade student results

III. CRITIQUE (5 min)

A. Questions From Students

B. Critique Test Items

SSG G.B. Howe
LP 08-280-617
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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

Other Parasitic Organisms of Medical Importance (2 hours)

COURSES PRESENTED TO: 311-92B10. Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, eraser, and magnetic placards (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

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I. INTRODUCTION (5 min)

A. Opening Statement: We have studied several of the most common organisms responsible for the clinical manifestations of parasitism in man. We must realize, however, that there are many other parasitic organisms which are not as frequently encountered, but which are of increasing importance and are often responsible for severe and debilitating diseases in man.

B. Objective: Realize that there are other parasitic organisms of medical importance.

C. Class Procedure and Lesson Tie-in: None

II. EXPLANATION (80 min)

A. Other Parasitic Protozoans of Medical Importance

1. Naegleria species

a. General Characteristics

(1) Facultatively parasitic amoeba

(2) Normally free-living in ponds and pools

(3) Morphological forms

(a) Trophozoite - amoeboid form

(b) Trophozoite - flagulated form - not found in clinical material

(c) Cyst

NOTE: Refer students to GR 08-280-601 for drawing

b. Geographic distribution - worldwide in ponds and pools

c. Habitat - brain and meninges

d. Specimen - CSF

e. Mode of Infection - penetration of nasal mucosa

f. Disease - meningoencephalitis

(1) destruction of brain tissue

(2) fatal in 2-3 days

g. Laboratory techniques

(1) Microscopic examination of CSF

(a) direct preparation

(b) stained smears

(2) Culture on artificial media

/

2. Ballantidium coli

- a. General characteristics
  - (1) Normally a ciliate of swine
  - (2) Only ciliate parasitic to man
  - (3) Morphological forms
    - (a) Trophozoite
    - (b) Cyst

NOTE: Refer students to GR 08-280-601 for drawings

- b. Geographic distribution - worldwide
- c. Habitat - large intestine
- d. Specimen - feces
- e. Mode of infection - injestion of mature cyst
- f. Disease - ballantidiasis
  - (1) Diarrhea
  - (2) invasion of intestinal mucosa
- g. Laboratory techniques - examination of specimen for trophozoites and cysts

3. Toxoplasma gondii

- a. General characteristics
  - (1) Normally an intestinal coccidian of cats
  - (2) Small, crescent-shaped intracellular parasite in man
- b. Geographic distribution - worldwide
- c. Habitat - nucleated cells
- d. Specimen - body fluids and tissue
- e. Mode of infection -
  - (1) injestion of oocysts
  - (2) Congenital

f. Disease - Toxoplasmosis

(1) Often asymptomatic

(2) Congenital infections often fatal

g. Laboratory techniques

(1) Serological techniques

(2) Stain and examine specimen for intracellular parasites

4. Leishmania species

a. General characteristics

(1) Small, oval, without flagella (Leishman-Donovan body)

(2) Usually intracellular

(3) Flagulated forms do not occur in man

(4) All mammilian Leishmanias may infect man

(5) General term for infection - Leishmaniasis

(6) Vector - sandfly

(7) Reservoir hosts - dogs, rodents, other mammals

b. Leishmania donovani

(1) Geographic distribution - Near East, Mediterranean area
South and Central America

(2) Habitat - visceral organs; spleen, liver, bone marrow

(3) Specimen - biopsy of effected organs, bone marrow impressions

(4) Mode of infection - bite of sandfly vector

(5) Disease - Kala Azar

(a) Called visceral leishmaniasis

(b) Mortality may be high in non-indigenous personnel

c. Leishmania tropica

(1) Gepgraphic distribution - Mediterranean area, South America, Asia

(2) Habitat - cells of cutaneous tissue

(3) Specimen - skin scrapings

(4) Mode of infection-

(a) direct contact

(b) bite of sandfly vector

(5) Disease - oriental sore

(a) Called cutaneous leishmaniasis

(b) Skin sores vary from small pimple to large ulcerated lesions

d. Leishmania braziliensis

(1) Geographic distribution - Central and South America, parts of Africa and Asia

(2) Habitat - mucous membranes of mouth, nose and tongue

(3) Specimen - biopsy material, exudate

(4) Mode of inf3ction -

(a) Direct contact

(b) Bite of sandfly vector

(5) Disease - espundia

(a) Called subcutaneous leishmaniasis

(b) Lesions resistant to treatment

e. Laboratory Techniques for Identification of Leishmania spp.

(1) Microscopic examianation of stained smears

z(2) Growth on artificial media

(3) Animal inoculation

5. *Trypanasoma* species
    - a. General characteristics
        - (1) Intra and extracellular parasites
        - (2) Morphological forms
            - (a) Flagulated, with undulating membrane; found in blood
            - (b) Oval, non-flagulated form; found in tissue
    - b. *Trypanasoma gambiense* and *Trypanasoma rhodesiense*
        - (1) Geographic distribution
            - (a) *T. gambiense* - west Africa
            - (b) *T. rhodesiense* - east Africa
        - (2) Habitat - lymph glands, spleen, CSF, blood
        - (3) Specimen - blood, CSF, biopsy material
        - (4) Mode of infection - bite of Tse Tse fly vector
        - (5) Diseases
            - (a) *T. gambiense* - West African sleeping sickness
            - (b) *T. rhodesiense* - East African sleeping sickness
    - c. *Trypanasoma cruzi*
        - (1) Geographic distribution- Central and South America, parts of southwest USA
        - (2) Habitat - cells of heart, voluntary muscles, and various glands, blood
        - (3) Specimen - blood, biopsy material
        - (4) Mode of infection - bite of reduviid bug vector (posterior station)

NOTE: Infective form of organism found in feces of vector

        - (5) Disease - Chagas disease

d. Laboratory techniques

(1) Microscopic examiantion of stained smears

(2) Growth on artificial media

(3) Animal inoculation

(4) Xenodiagnosis

SECOND HOUR

B. Other Parasitic Helminths of Medical Importance

1. Echinococcus species

a. General characteristics

(1) Cestodes - adult normally intestinal parasites of canines

(2) Man is an accidental intermediate host

(3) Hydatiform cysts often found in sheep

(4) Hydatid cysts formed in various organs in man

b. Geographic distribution - worldwide in sheep raising countries

c. Habitat- larvae in various organs
d. Specimen - biopsy material, exudate
e. Mode of infection - accidental injestion of eggs

f. Diseases

(1) *E. granulosus* - Echinococcosis, hydatid disease

(2) *E. multilocularis* - multilocular cysts with external budding; invade other tissues

g. Laboratory techniques

(1) Serological tests

(2) Examination of hydatid fluid and tissues for scolices and brood capsules

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2. Trichinella spiralis

a. General characteristics

(1) Nematode - found in swine, rodents, other mammals

(2) Larvae in muscle tissue

b. Geographic distribution - worldwide

c. Habitat

(1) Adult - small intestine

(2) Larvae - striated muscle tissue

NOTE: Man may harbor either the adult or the larval form. Infection with adult form is rarely of clinical signifigence

d. Specimen - muscle biopsy

e. Mode of infection - ingestion of encysted larva in muscle of poorly cooked pork

f. Disease - trichinosis (caused by larval form)

g. Laboratory techniques

(1) Serological tests

(2) Examination of biopsy material for larval forms

3. Filarial worms

a. General characteristics

(1) Nematodes

(2) Larval forms called microfilaria

(3) Microfilaria exhibit nocturnal periodicity

b. Geographic distribution of common species

(1) Wuchereria bancrofti - warm countries, central Japan, parts of Southwest USA

(2) Brugia malayi - Asia

c. Habitat

(1) Adult - lymphatics

(2) Microfilaria - blood

d. Specimen - blood

e. Mode of infection - bite of mosquito vector

f. Disease - elephantiasis

g. Laboratory techniques

(1) Draw blood between 2200 and 0200 hrs for best

NOTE: This increases the chances for recovery due to the nocterral periodicity of the microfilaria

(2) Concentration - Knott's technique

(3) Observe for microfilaria in concentrated blood

III. SUMMARY (5 min)

A. Review of Main Points

1. Other parasitic protozoans of medical importance

2. Other parasitic helminths of medical importance

B. Closing Statement: Most of the parasites we have discussed are rarely encountered in routine laboratory situations. It is important, however, that the laboratory technician be aware that parasitic infections by the more bizarre organisms do occur in a variety of specimen types.

INSTRUCTIONAL HOURS

Number of instructors needed - - - 2

Primary hours 2

Secondary hours 0

TOTAL 2

SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-619
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

Final Scan (3 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: All previous references

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Microscope, lamps, prepared microscope slides of organisms studied (see Annex A), immersion oil, and lens paper.

PERSONNEL: One instructor (MOS 92B3H) per six students

INSTRUCTIONAL AIDS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical Exercise 1

I. INTRODUCTION (5 min)

A. Opening Statement: Your ability to perform in a parasitology section of the clinical laboartory depends in a large measure on your ability to scan microscopic slides and identify select parasitic organisms present. This examination is designed to test your ability to perform this most important task.

B. Objectives

1. Given stained blood smears, microscopically locate and determine the presence of the genus Plasmodium to report whether the smear is positive or negative for malaria with an accuaracy of 75 %.

2. Microscopically locate and identify from prepared slides, identification forms representing all classes studied with an accuracy of at least 70 %.

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*This lesson plan supersedes LP 08-280-619, 017.

C. Class Procdeure and Lesson Tie-in

None

II. APPLICATION (105 min)

A. FIRST HOUR - Malaria Scan

1. Prior to class, final scan microscopic sets are prepared and keyed (see Annex A)

2. Arrangement of students

3. Directions to students

   a. Notes allowed

   b. No talking

   c. Time allowed: 1 hour

   d. Record results on answer sheet (M 08-280-619-1)

4. Students' activities

   a. Scan malaria slides

   b. Determine whether malarial parasites are present or absent

   c. Record result on answer sheet (M 08-280-619-1)

5. Instructors' activities

   a. Pass out final scan sets and answer sheet (M 08-280-619-1)

   b. Grade student results

B. SECOND AND THIRD HOURS - Helminth and Protozoan Scan

1. Prior to class, final scan microscopic sets are prepared and keyed (see Annex A)

2. Arrangement of students

3. Directions to students

   a. Notes allowed

b. No talking

c. Time allowed: 2 hours

d. Record results on answer sheet (M 08-280-619-1)

4. Students' activities

a. Scan slides for protozoan cysts and helminth ova and larva

b. Results should be reported to genus and species is applicable on report sheet (M 08-280-619-1)

5. Instructors' activities

a. Pass out final scan sets and answer sheet (M 08-280-619-1)

b. Grade student results

III. REVIEW (5 min)

A. Review of Main Points

B. Reteaching of Students

C. Closing Statement: Your demonstrated proficiency on this examination is evidence of the fact that you can identify the parasitic organisms learned in this course to the required standards.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1 per 6 students

Primary hours	3
Secondary hours	0
TOTAL	3

in water and passing it through a #30 to #60 sieve, and then searching for adults on the sieve with a hand lens.

In general, the sooner a specimen can be examined following passage the more reliable the results will be. If several specimens are received in the laboratory at the same time, examine the watery, loose, and soft stools before examining the formed ones. Fluid stools may contain trophic forms which will soon perish. There should be no undue delay in examining formed stools if delay can be avoided inasmuch as motile organisms may occur on their outer surfaces. If for any reason formed stools must be retained in the laboratory for an appreciable length of time before examination, they may be stored in the refrigerator at ordinary refrigerator temperatures for periods up to several days. Formed stools are likely to contain cysts and eggs primarily and these will be well preserved by refrigeration. Fluid stools received in fresh condition may be stored in the incubator. This storage should not exceed 30 minutes because increased bacterial multiplication quickly destroys motile organisms.

In numerous situations it may be necessary to forward fecal specimens to a laboratory through the mail. Smaller laboratories, particularly those at dispensaries, may either desire or be required to forward specimens upon which certain diagnoses have been made to larger central laboratories for confirmation. In survey work it often becomes necessary to mail fecal specimens. Certain postal regulations must be observed. These require the use of a double container in which the receptacle containing the specimen is placed. The specimen can be introduced into a round one-ounce sputum shell vial and corked; however, the screw-cap bottles are preferable and should be used when available. The vial or bottle in turn is suspended in cotton packing in a metal, screw-cap container and the latter is shipped in a standard cardboard and metal, screw-top mailing tube. The above method of packing meets all postal requirements. The vial containing the specimen should not be filled to capacity because rapid fermentation, especially in hot weather, will result in gas production and the cork may blow off. Do not add urine or water to the specimen. Similarly, disinfectants and preservatives should not be added inasmuch as they interfere with flotation techniques. A slip with necessary data concerning the source of the specimen together with any special instructions should be wrapped around the inner metal container. The specimen should be mailed on the same day it is passed.

If adult worms or parts thereof are forwarded for identification, they should be washed free of all debris and placed in 70 percent alcohol or 10 percent formalin for shipment. In the section which follows, the polyvinyl alcohol (PVA) method for preservation and shipment of fecal specimens described is particularly applicable to shipment of specimens known or suspected to contain trophic amoebae.

Inaccurate or incomplete identification of specimens can lead to grave error in the laboratory. Where appreciable numbers of specimens are received a routine procedure which has been found very workable is to assign a number to each specimen for each day's work. This number is affixed to each slide, tube, or other device to which the specimen is transferred during examination.

Standard-sized slides tend to increase the possibility of contamination. Larger slides, 1½ x 3 inches or 2 x 3 inches are preferable for temporary mounts. Toothpicks, applicator sticks or other implements used to transfer or mix fecal suspensions should be used only once. All objects which come into contact with fecal specimens, i.e., applicators, slides, coverslips, and the like, should be placed immediately in a solution of 5 percent phenol or other suitable disinfectant after use. Always avoid the possibility of infecting yourself or others by carefully handling and disposing of all objects with which fecal specimens come in contact.

In the preparation of temporary mounts such as saline, iodine, or vital stained preparations, use only a small amount of feces. The total quantity of fluid under the coverslip should not exceed about three drops. It is particularly important that inexperienced technicians prepare rather thin preparations. With increased experience thicker preparations can be used effectively. Always coverslip temporary preparations to avoid the possibility of contaminating microscope objectives. In applying coverslips do not exert excessive pressure. Doing so will force the fluid suspension out and also tends to crush ova which may be present. Certain precautions must be taken in the handling of fecal suspensions which are centrifuged at any stage in their processing. Make certain that the centrifuge is properly balanced and that the mouths of the tubes are plugged in such a manner that the plugs will not be thrown down into the tubes. Centrifuges contaminated as a result of breakage of tubes or splattering due to entry of plugs into their contents are difficult to disinfect properly.

When the presence of *E. histolytica* is suspected but stools are negative, positive specimens may be obtainable from ulcerative lesions in the rectum or sigmoid. These specimens are obtained through a proctoscope or sigmoidoscope by the physician. Arrangements should be made to have laboratory personnel present to receive the specimen and take it to the laboratory for immediate examination.

Examination of material from a single stool specimen undoubtedly results in the missing of many parasitic infections. With many parasites the passage of stages of the life cycle in the fecal mass is irregular. It can only be concluded from all available evidence in the numerous studies on rate of parasite detection that a minimum of three stools should be examined on each patient preferably on alternate days. These should be followed by examination of a purged stool if prior results are negative. The latter procedure is particularly applicable to fecal examinations in cases of suspected infection with *Endamoeba histolytica.* However, in some cases cyst passage is so sporadic that even three consecutive examinations may fail to reveal infection with this organism.

PREPARATION AND EXAMINATION OF TEMPORARY MOUNTS

Following macroscopic examination, the next step in the routine examination of a fecal specimen should be the preparation of two temporary mounts on the same slide. These consist of a saline suspension and an iodine stained suspension on opposite ends of the slide. The saline suspension is prepared first, and then with the same applicator stick a portion of the specimen is transferred to the iodine stain. Fecal samples should be taken from several portions of soft and formed stools. The more heterogeneous the stool specimen, the more samples should be taken. If flecks of mucus or blood occur on the outside surface of formed stools these should be selected for examination. When trophozoites are suspected in fresh preparations various techniques may be employed to keep the specimen warm during examination. Both the slides and saline may be placed in a 37° C. incubator prior to preparation of the mount. Slides should not be warmed on microscope lamps inasmuch as temperatures attained are difficult to control or ascertain. Commercial type warm stages are excellent but not absolutely essential for examination for trophic forms. Placing a heated penny on the saline suspension end of the slide helps to keep the preparation warm during examination. It should also be pointed out that in saline preparations time must be allowed for the trophic amoebae to settle down and commence their amoeboid activities. The trophic amoebae will be rounded up and immotile if the preparation is examined too soon. Some workers prefer to make the saline mount, apply a coverslip ringed with petrolatum, and warm the preparation in the incubator for about 5 minutes before examining for motile organisms.

Temporary mounts should be searched systematically and the entire preparation should be examined. It is good practice to utilize low power, switching to the high dry objective for a more careful examination of any objects suggestive of parasites. If no organisms can be found with the low power objective a short final search should be made under high dry magnification to avoid overlooking smaller organisms which might otherwise be missed.

The search should be orderly, starting at the top right hand corner (because of inversion of the image this is actually the lower left) and moving back and forth across the preparation until the entire area under the coverslip has been searched.

One of the major causes of failure to detect parasites in feces is the failure to maintain proper illumination. Illumination must be constantly adjusted with each change of magnification and with each object which is observed for morphology. The distance between condenser and stage as well as the size of the opening in the iris diaphragm need constant adjustment to accentuate details of objects observed. Too much light renders objects invisible while too little light obscures morphologic details.

Direct Wet Smear

It cannot be emphasized too strongly that the *very first step* in the microscopic examination of a fecal specimen for parasites should be the preparation and examination of a direct wet smear. This is the easiest direct method for screening specimens submitted to the laboratory for examination. The unstained portion of the mount will contain the trophic forms, when present, while the iodine stained portion of the mount facilitates detection of organisms in the cystic stage and allows for detailed study of their internal morphology. Ova can be readily observed in either the iodine-stained or saline portion of the preparations.

Materials and Equipment:

- Medicine dropper
- Physiological saline (54)
- Coverslips
- Slides
- Iodine solution (39)
- Applicator sticks

Technique:

1. With a medicine dropper place a drop of physiologic saline in the center of one end of the slide and a drop of any of the various standard iodine stains in the center of the opposite end.
2. With a wooden applicator stick, select small portions of feces from various parts of the fecal mass. If the stool contains blood or mucus, these portions should be selected.
3. Mix and spread a small quantity of the specimen evenly with a wooden applicator on the saline portion of the preparation. Similarly mix and spread a small portion of the material on the iodine side of the preparation. Proper density of the smears is indicated when newspaper print can barely be read through the preparation.
4. Apply coverslips to the two smears and examine under the low power objective switching to high dry for observation of details of suspicious objects. Commencing at one corner of each temporary mount, both of the preparations should be systematically and completely searched.

Iodine Stains

Methods of preparation of iodine stains and the duration of their effectiveness after preparation are enumerated elsewhere. The necessity of utilizing stain of proper age, and stain which will give proper intensity of contrast cannot be overemphasized. Stain which is too concentrated is absorbed so rapidly that the entire cyst assumes a uniform dark brown hue. Stains which are too low in concentration are not absorbed sufficiently and the cysts tend to blend in with their surroundings rendering them invisible against the similarly colored lemon-yellow background. In old stains there is a tendency for the iodine to become sublimated. Also, if the stain is left on the slide too long before the feces is mixed with it, uneven staining, as well as over-staining, is likely to result.

Vital Stains

The nuclei of trophic amoebae are indistinct or entirely invisible in saline preparations. Since solutions containing iodine destroy trophic organisms immediately they are unsatisfactory for demonstrating living organisms.

These difficulties have been overcome by the development of suitable vital stains, two of which will be described here.

Staining with Quensel's Solution:

Materials and Equipment:

Medicine dropper
Quensel's solution (57)
Slides
Coverslips
Applicator sticks

Technique:

1. With a medicine dropper transfer a drop of Quensel's solution to the center of a glass slide.
2. Pick up a small amount of the fecal specimen with an applicator stick. Transfer it to the drop of stain on the slide and mix thoroughly.
3. Coverslip and let the preparation stand for 10-20 minutes to allow permeation of the organisms by the staining solution. Slides should be placed in a 37° C. incubator during this interval.
4. Following staining examine the preparation under high dry magnification. Cytoplasm will be stained a pale blue and the nuclei a deeper shade of blue. Stained nuclei present the same morphological characteristics as in permanent hematoxylin preparations. The nuclei of *Dientamoeba fragilis* do not stain well but the presence of 1 or 2 nuclei is clearly discernible. Ciliates, flagellates, and cysts do not stain. After 1-2 hours the motile organisms become overstained and nuclear morphology can no longer be discerned.

Staining with Velat, Weinstein, and Otto Solution:

Materials and Equipment:

Medicine dropper
Velat et al's. solution (70)
Slides
Coverslips
Applicator sticks

Technique:

1. With a medicine dropper transfer one or two drops of the staining solution to the center of a glass slide.
2. With an applicator stick pick up a small amount of the fecal specimen, transfer it to the stain and mix thoroughly.
3. Let stand for 5 minutes in a 37° C. incubator and examine. Critical staining time is about 5 minutes at pH range of 4.6–4.8. At higher pH ranges staining time is from 5–15 minutes. There is less tendency for organisms to overstain at higher pH range.
4. The nuclei and karyosome bodies of all the amoebae stain purple-black, and the cytoplasm a light purple.
5. Flagellates usually become immobilized and tend to swell to some extent but characteristic structures of the various species are usually easily observed. The chromatin of the nucleus of *Chilomastix mesnili* stains a light purple. The nuclear membrane, fibrils, and flagella of *Giardia lamblia* stain a purple-black while the cytoplasm stains a light purple-pink color.

Merthiolate-Iodine-Formalin Staining of Protozoan Cysts (MIF) in Fresh Fecal Specimens (Sapero, Lawless, and Strome)

This is a simple rapid technique useful for accentuating morphology of cysts. Added to fresh fecal specimens it also serves as a preservative in which specimens can be shipped or stored. The staining reaction with this solution occurs in two phases, an initial iodine phase in which cysts are stained yellow-brown followed by an eosin phase in which the cytoplasm of cysts takes on a pinkish hue. Nuclei stain dark red to jet black. The nuclear elements of all human protozoan cysts are well defined.

Materials and Equipment:

Medicine dropper
Distilled water

Slides
Coverslips
MIF solution (prepare a fresh staining solution once each week) (51)
Applicator sticks

Technique:

1. With a medicine dropper place a drop of distilled water on a slide and add an equal amount of the MIF stain.
2. To this mixture add a small portion of the fecal specimen and mix thoroughly.
3. Coverslip and allow the preparation to stand for a few minutes to permit permeation of cysts by the staining solution.
4. Examine under low power and confirm findings under high dry magnification.

Concentration Techniques

Zinc Sulfate Centrifugal Flotation (Faust). This is a very efficient technique for the detection of helminth ova, larvae, and adults of some of the smaller nematodes. It is also useful for the recovery of protozoan cysts. Fluke and tapeworm eggs bearing opercula, and the nonoperculate ova of the schistosome flukes are not generally recovered by this method. The method is economical and rapid and is recommended as a routine procedure in medical laboratories.

Materials and Equipment:

Applicator sticks
Test tube, 15 ml
Funnel, 50 mm
Gauze
Wassermann tube
Zinc sulfate solution, specific gravity 1.18–1.20 (75)
Test tube rack
Slides
Coverslips
Loop, 5 mm attached to holder (Plane of the loop bent at right angle to the wire)
Iodine solution (39)

Technique:

1. Using two applicator sticks, remove a sample of feces about the size of a pecan. Place the fecal specimen in a test tube containing about 10 ml of tap water and mix very thoroughly with the applicators.
2. Strain this suspension through a 50 mm funnel lined with two layers of wet gauze into a Wassermann tube filling it two-thirds full.
3. Centrifuge this preparation for 1 minute at about 2,500 rpm. This step throws ova, cysts, and other heavier particles to the bottom of the tube, while oils and lighter debris remain in the supernatant.
4. Pour off the supernate fluid and add a small amount of tap water to the tube and break up the sediment with an applicator stick. After breaking up the sediment, fill the tube two-thirds full with tap water once more.
5. Again centrifuge at 2,500 rpm for 1 minute repeating this washing procedure until the supernatant is relatively clear. (Usually a total of 3 washings suffices.)
6. Decant the supernate and add sufficient zinc sulfate solution to fill the tube half full. With an applicator stick break up the packed sediment very thoroughly, then add zinc sulfate solution to fill the tube to within an inch of the top.
7. Centrifuge again for 1 minute at 2,500 rpm. The latter step causes certain parasite ova, larvae, cysts, and some adults to float to the top. Heavier fecal debris will be thrown to the bottom.
8. Carefully remove the tube from the centrifuge and without agitating the contents place it in vertical position in a rack.
9. Wait 1 or 2 minutes, then touch a wire loop of approximately 5 mm diameter to the surface film removing several loopfuls in this manner to a glass slide. It is important that the loop be parallel

to the surface film and the holder held perpendicular to the film.

10. Add a drop of iodine solution, mix thoroughly, and coverslip. Systematically search the entire mount with the low power objective switching to high dry for observation of details of suspicious objects.

Modified Zinc Sulfate Centrifugal Flotation. This modification of Faust's original method saves time and equipment since the straining of the fecal suspension through gauze is omitted, and washing by centrifugation is performed only once instead of 2 or 3 times. Removal of the flotage by superimposed coverslip also simplifies the technique.

Materials and Equipment:

Applicator sticks
Wassermann tube
Zinc sulfate solution, specific gravity 1.20 (75)
Test tube rack
Slides
Iodine solution (39)
Coverslips

Technique:

1. Using 2 applicator sticks, remove a sample of feces about 2 ml in volume. Place the fecal specimen in a Wassermann tube one-half filled with tap water and comminute very thoroughly, breaking up all obvious particles.
2. Centrifuge this preparation for 1 minute at approximately 2,500 rpm.
3. Pour off the supernatant fluid and repeat this washing only if the stool is extremely oily. Add enough zinc sulfate solution to fill the tube one-half full and then with an applicator stick break up the packed sediment very thoroughly.
4. Add zinc sulfate to fill the tube to within an inch of top and centrifuge again at 2,500 rpm for 1 minute.
5. Without shaking or spilling, carefully place the tube in vertical position in a rack and then slowly add zinc sulfate solution until the tube is brimful.
6. Place a drop of iodine solution on a slide.
7. Wait 1-2 minutes. Then deftly touch a clean, grease-free coverslip to the meniscus, and gently remove the coverslip with a straight upward motion.
8. If ova or cysts are present they will be contained in the drop which adheres to the underside of the coverslip. Lower the coverslip onto the drop of iodine stain on the slide and examine. Systematically search the entire preparation with the low power objective switching to high dry for observation of details of suspicious objects.

Zinc Sulfate Levitation (Otto). This technique is especially useful for the recovery of hookworm ova in survey work where large numbers of specimens are being examined. Recovery of cysts by this method is also good, but not as satisfactory as zinc sulfate centrifugal flotation techniques.

Materials and Equipment:

Applicator sticks
Vial, approximately 2 inches in height and ¾ inch in diameter
Zinc sulfate solution, specific gravity 1.18 (75)
Test tube rack
Slides
Coverslips
Iodine solution (39)

Technique:

1. Using 2 applicator sticks, remove a sample of feces about the size of a kidney bean and place it in a vial approximately 2 inches high and ¾ inch in diameter.
2. Fill the tube one-half full with zinc sulfate solution and comminute very thoroughly with the applicator sticks.
3. Place the tube in a test tube rack making sure the tube is perpendicular.

4. Fill the tube brimful with zinc sulfate solution. Carefully apply a coverslip which is larger than the diameter of the tube to the surface of the liquid. There should be no air pockets beneath the coverslip and no overflow of fluid.
5. Allow the preparation to stand for 1 hour. Remove the coverslip with a straight, upward motion and move it to a slide on which a drop of iodine solution has been placed.
6. Examine the preparation under low power covering the entire mount. Cysts and ova should be confirmed under high dry.

Brine Flotation (Willis-Malloy). This is a simple, extremely efficient technique for recovery of nonoperculate ova, except for those of the schistosome flukes. It is of no value for recovery of protozoan cysts since they will be unrecognizably shrunken. It would be most valuable, for example, in rapid screening tests for nematode infections in large numbers of individuals.

Materials and Equipment:

Applicator sticks
Vial, about 1 inch in diameter, capacity approximately 20 ml
Sodium chloride, saturated solution, specific gravity 1.20
Slides
Coverslips

Technique:

1. Using 2 applicator sticks, remove a sample of feces about the size of a marble. Place the specimen in a 1-inch diameter 20 ml vial and fill one-fourth full with salt solution.
2. With the applicator sticks comminute the specimen until the feces is suspended as a fine silt.
3. Fill the vial to the brim with salt solution and then carefully superimpose a glass slide to bring its undersurface into contact with the meniscus. There should be no air pockets beneath the slide and no overflow of fluid.
4. Allow the slide to remain in contact with the meniscus of the fluid for 10 minutes to 1 hour. Optimum time is about 15 minutes.
5. Remove the slide by lifting it straight up without tipping. If ova are present they will be contained in the drop which adheres to the undersurface of the slide. Deftly invert the slide without allowing the drop to run off in the process.
6. Coverslip and examine under low power. Confirm findings under high dry. The preparation should be examined immediately and before the mount commences to dry.

Acid-Ether Technique (Telemann). This is a method which is useful for the concentration of all helminth ova except those which bear opercula. It is especially good for the detection of the ova of *Schistosoma mansoni* and *Schistosoma japonicum.* Protozoan cysts are damaged by the acid and this is not a satisfactory method for their recovery. The procedure utilizes an acid to dissolve albuminous matter, soaps, mucin, phosphates and various calcium salts. Ether dissolves the neutral fats and fatty acids. These actions together with mechanical agitation free the ova from the detritus in which they are entrapped. Embryos are killed by the reagent and in schistosomiasis infections, therefore, effectiveness of treatment cannot be determined by this method. It should also be pointed out that the method is expensive when used routinely due to the cost of ether.

Materials and Equipment:

Applicator sticks
Test tube, 20 ml
Hydrochloric acid, 15 percent solution
Aqueous Triton N E, 10 percent solution (if available)
Gauze
Funnel, 50 mm diameter
Centrifuge tube, graduated, 15 ml
Ether
Stopper for centrifuge tube
Slides
Coverslips
Pipette, capillary with rubber bulb

Technique:

1. Using 2 applicator sticks, remove a sample of feces about the size of a marble. If schistosomiasis is suspected, try to secure this entire sample by scraping the outer surface of the stool specimen.
2. Place the fecal specimen in a 20 ml test tube containing 5–10 ml of a 15 percent HCl solution and mix very thoroughly with the applicators. If the reagent is available, it is helpful to add 0.6 ml of Triton X E. This will enhance release of ova from the fecal debris.
3. Strain 5 ml of the fecal suspension through 2 layers of wet cheesecloth or gauze contained in a 50 mm diameter funnel, into a 15 ml graduated centrifuge tube and add an equal quantity of ether.
4. Insert a rubber stopper and shake thoroughly for about 1 minute. Remove the stopper and centrifuge for 1 minute at 1,500 rpm.
5. Four layers should result: a small amount of sediment remaining in the bottom of the tube containing most of the ova, a layer of acid, a plug of detritus just on top of the acid, and a topmost layer of ether.
6. With an applicator stick, ring the plug of debris to free it from the sides of the tube. Quickly and carefully decant the top three layers leaving the sediment undisturbed.
7. Do not rotate the tube back to a vertical position but keep it horizontal. This prevents materials adhering to the side of the tube from flowing back and diluting the bottom sediment.
8. With the tube in horizontal position, insert a long capillary pipette with rubber bulb attached, into the sediment and stir it before withdrawing some into the pipette. Discharge on a clean slide, coverslip, and examine under low power. Confirm findings under high dry.

Formalin Ether Sedimentation Technique (Ritchie). In large scale usage this technique has been ascertained to be excellent for the recovery of the cysts of protozoa and it is also very good for the recovery of helminth eggs.

Materials and Equipment:

Physiologic saline (54)
Beaker, 150 ml
Gauze
Funnel, 50 mm
Pointed centrifuge tubes, 15 ml
Formalin, 10 percent solution
Ether
Stopper for centrifuge tube
Applicator sticks
Pipette, capillary, with rubber bulb
Coverslips
Slides
Iodine solution (39)

Technique:

1. Comminute a portion of stool about the size of a walnut in 10–12 ml of saline in a beaker. Mix thoroughly and pass the mixture through a funnel lined with 2 layers of wet gauze into a 15 ml pointed centrifuge tube.
2. Centrifuge at 1,500–2,000 rpm for 2 minutes. Decant the supernate and resuspend the sediment in fresh saline, centrifuging and decanting as before. This operation is repeated until the supernate is of the desired clarity.
3. Following final decanting add about 10 ml of 10 percent formalin to the sediment, mix thoroughly, and allow to stand for 5 minutes.
4. Add 3 ml of ether, stopper the tube, and shake until thoroughly mixed.
5. Centrifuge at 1,500 rpm for about 2 minutes. Four layers should result: a small amount of sediment containing most of the protozoan cysts and ova, a layer of formalin, a plug of detritus just on top of the formalin, and a topmost layer of ether.
6. With an applicator stick ring the plug of debris to free it from the sides of the tube. Quickly and carefully

decant the top three layers leaving the sediment undisturbed.

7. With an applicator stick mix the sediment remaining in the bottom of the tube with the fluid that drains back from the sides. With a pipette with attached rubber bulb transfer a drop of sediment to a slide, add a drop of iodine and mix thoroughly. Coverslip and examine under low power. Confirm findings under high dry.

Sedimentation. This simple technique is applicable to recovery of all ova, cysts and small nematodes. It is a combination of straining and sedimentation which is most useful for recovery of operculate and schistosome ova. Since it is a washing process, concentration, especially in light infections, is not very marked and preparations usually contain much confusing debris. The use of a large fecal sample, however, helps to offset the latter disadvantage.

Materials and Equipment:

Tongue depressor
Beaker, 250 ml
Glycerin, 0.5 percent solution
Funnel, 50 mm
Gauze
Glass, sedimentation or graduate, cone-shaped
Pipette, capillary, with rubber bulb attached
Slides
Coverslips
Iodine solution (39)

Technique:

1. With a tongue depressor remove a sample of feces about the size of a walnut and place it in a 250 ml beaker. Fill the beaker one-fourth full with a 0.5 percent solution of glycerin in tap water. Tap water may be substituted but glycerinated water tends to reduce surface tension resulting in greater yield of ova.
2. With a tongue depressor mix feces very thoroughly with the diluent, then add sufficient diluent to fill the beaker three-fourths full.
3. Strain this suspension through a 50 mm glass funnel lined with 2 layers of wet gauze draining the fluid into a conical sedimentation glass or cone-shaped graduate.
4. Allow the suspension to settle for 1 hour and then carefully pour off two-thirds of the supernatant fluid without allowing the sediment to escape.
5. Add fresh diluent and break up the sediment thoroughly to resuspend the sediment uniformly throughout the fluid. Allow settling for an additional hour. The washing procedure should be repeated until the supernatant fluid is relatively clear. However, one washing will generally suffice.
6. Carefully pour off the last wash diluent without losing any of the sediment.
7. Utilizing a capillary pipette with rubber bulb attached remove successively several drops of sediment from the top, center and very bottom of the sediment and transfer to glass slides. Coverslip and examine under low power. Confirm findings under high dry.

Note: If a specimen has been previously washed, strained, and fixed in formalin take 10 ml of the thoroughly mixed specimen and start at step 4.

Merthiolate-Iodine-Formaldehyde-Concentration Technique (MIFC) (Blagg et al.):

Materials and Equipment:

Merthiolate-Iodine-Formalin solution (M.I.F.) (51)
Applicator sticks
Gauze
Centrifuge tube with rubber cork
Ether
Slides
Coverslips

Technique:

1. With an applicator stick mix thoroughly 1 part of the fecal suspension with 3 parts M.I.F. solution.

Preserved in this manner specimens can be stored and examined later.

2. To concentrate the specimen prior to examination, strain this mixture through 2 layers of wet gauze into a 15 ml centrifuge tube.
3. Add 4 ml of ether to the tube, insert a rubber stopper and shake the tube vigorously. (If ether remains on top after shaking add 1 ml of tap water and reshake.)
4. Remove stopper and let stand for about 2 minutes.
5. Centrifuge for 1 minute at 1,600 rpm. Four layers will appear in the tube: an ether layer on top, a plug of fecal detritus, an M.I.F. layer, the sediment containing protozoa and helminth ova on the bottom.
6. Loosen the fecal plug by ringing with an applicator stick.
7. Quickly, but carefully, pour off all but the bottom layer of sediment.
8. Thoroughly mix this sediment, pour a drop on a slide, coverslip and examine.

Centrifugation. All helminth ova including those of the schistosomes and cysts can be recovered by this technique. It is thus useful on all specimens regardless of what parasites may be present. Other advantages of this method are that it is rapid, reveals embryos in living condition, does not require expensive reagents to perform, and enhances detection of parasites in low grade infections since a large quantity of feces is used.

Materials and Equipment:

Tongue depressor
Test tube, 25 x 150 mm
Stopper, rubber, No. 4
Gauze
Funnel, 50 ml
Tube, centrifuge, 50 ml
Pipette, capillary, with rubber bulb attached
Slides
Coverslips
Iodine solution [3] (39)

Technique:

1. With a tongue depressor remove a sample of feces about the size of a walnut. Utilization of a large sample is one of the principal advantages of this technique.
2. Transfer the specimen to a 25 x 150 mm test tube half filled with water and with a tongue depressor mix very thoroughly.
3. Fill the tube two-thirds full with tap water and insert a No. 4 rubber stopper. Shake the tube vigorously for at least 1 minute.
4. Pour the suspension through a 50 mm funnel lined with 2 layers of wet gauze into a 50 ml centrifuge tube. Spin the specimen for 1 minute at a speed of 1,000–1,500 rpm. To avoid collapse of ova, maximum speed indicated here should not be exceeded.
5. Pour off the supernatant fluid. Using a long capillary pipette with rubber bulb attached, take up a few drops of the top of the sediment and transfer it to the center of one end of a slide. Next insert the pipette to the bottom of the sediment removing a few drops. Transfer this sediment to the opposite end of the slide.
6. Coverslip the two preparations, and examine both mounts under low power. Confirm findings under high dry.

Note: Make a second mount and stain with iodine if cysts are observed in first preparation. Iodine is omitted in initial examination to avoid killing of miracidia and embryos.

Centrifugation for Recovery of Ova from Urine

Ova of *Schistosoma haematobium* are most generally voided in the urine. The last 15–20 ml of urine voided will most often contain the eggs.

Materials and Equipment:

Beaker, 50 ml
Tube, centrifuge, 15 ml

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[3] Used only on second mount when cysts are observed on initial examination.

Pipette, capillary, with rubber bulb attached
Slides
Coverslips

Technique:

1. Collect the last 15–20 ml of urine voided into a clean beaker.
2. Transfer urine to a 15 ml centrifuge tube filling it three-fourths full. Centrifuge 1 minute at 1,000–1,500 rpm.
3. Pour off the supernatant fluid. With a capillary pipette fitted with rubber bulb remove several drops of the sediment and transfer to a slide.
4. Coverslip and examine for ova under low power. Confirm findings under high dry.

Egg Hatching for Schistosome Miracidia

In fecal samples containing small numbers of viable eggs of schistosomes, the eggs may be overlooked when routine methods of examination are employed. The procedure here described is especially sensitive and is useful in demonstrating indirectly the presence of schistosome ova when few are present.

Materials and Equipment:

Beaker, 250 ml
Chlorine-free water
Tongue depressor
Funnel, 50 mm
Gauze
Glass, sedimentation or graduate, cone-shaped
Flask, Erlenmeyer, 500 ml
Applicator sticks
Slides
Coverslips

Technique:

1. Comminute a sample of feces about the size of a large walnut in a 250 ml beaker filled one-fourth full with chlorine-free water and mix thoroughly with tongue depressor. The sample should be obtained by scraping the outer surfaces of the fecal mass.
2. Add sufficient water to fill the beaker three-fourths full. Strain this suspension through a funnel lined with 2 layers of wet gauze into a sedimentation glass or cone-shaped graduate and allow the suspension to settle for 1 hour.
3. Carefully pour off the supernatant fluid without allowing the sediment to escape. Add a small quantity of water to the sediment and resuspend it with an applicator stick. Pour the suspension into a 500 ml Erlenmeyer flask and fill it almost to the lip with water. Allow to stand at room temperature overnight.
4. Hatched miracidia will swim about and collect in the neck of the flask thus concentrating them in a small area where they will be more easily visible. Against a dark background the miracidia will be observed as minute, white organisms swimming rapidly in a straight course. Use of a hand lens is helpful.
5. Free living ciliates are easily confused with miracidia. Identification should, therefore, be confirmed by transferring a few drops of fluid containing the organisms to a glass slide. Coverslip and examine. To stop motion of organisms add weak iodine solution or dilute methylene blue. This will enable careful study of the organisms.

Techniques for Recovery of Ova of Enterobius Vermicularis

Ova of pinworms are rarely encountered in routine fecal examinations since the female migrates to the perianal region to deposit ova. Several techniques have been developed for recovery of ova of this species. The basic principle in all of them consists in utilization of Scotch type cellophane tape to which eggs adhere when the sticky side is appressed to the skin in the perianal region.

The NIH Technique (National Institutes of Health):

Materials and Equipment:

Scotch type cellophane tape
Test tube fitted with a cork stopper through which a small glass rod (approximately 3 inches in length) is inserted
Rubber band
Glass slide

Technique:

1. Attach a piece of cellophane tape about 1 inch square, sticky side outermost, to the end of a glass rod with a rubber band. The glass rod is passed through a cork which is inserted into a test tube in transit.
2. To obtain the specimen appress the cellophaned tip of the glass rod to several areas in the perianal region.
3. To examine the specimen remove rubber band to free the cellophane paper and spread the paper out smoothly on a glass slide with the sticky side of the paper toward the slide.
4. Examine under low power and confirm findings under high dry.

The CDC Technique (Communicable Disease Center):

Materials and Equipment:

Scotch type cellophane tape, about three-fourths inch in width
Tongue depressor
Glass slide
Gauze or cotton

Technique:

1. Place a piece of Scotch type cellophane tape about three-fourths inch in width and about 2 inches in length on a standard sized glass slide, sticky side against the slide, and allow about one-half inch of the tape to overlap the slide at one end.
2. Just prior to taking the specimen hold a tongue depressor flat against the untaped side of the slide with the tongue depressor protruding about 1 inch below the end where the Scotch tape overlaps. Lift long portion of tape from the slide and loop it over end of depressor to expose gummed surface.
3. Hold tape and slide against tongue depressor with the right hand.
4. Spread the buttocks with the left hand and press gummed surfaces against several areas of the perianal region.
5. Replace tape on slide.
6. Smooth tape with cotton or gauze.
7. Examine under low power and confirm findings under high dry.

Note Specimens are best obtained a few hours after the individual has retired or the first thing in the morning before a bowel movement or bath. Details of the proper method of obtaining the specimen are shown in figure 1-1.

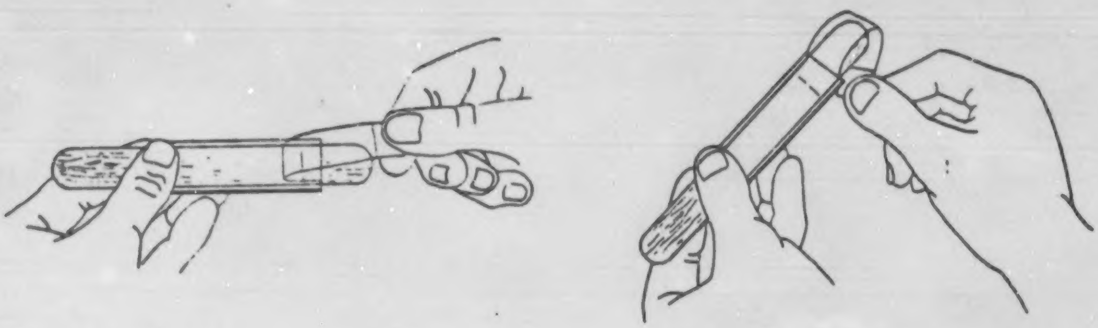
PVA-Fixative Techniques

PVA-fixative techniques were developed primarily as a method of preserving trophozoites of intestinal amoebae. Trophozoites disintegrate rapidly after passage from the host and it is, therefore, often difficult to obtain laboratory confirmation of suspected cases of amoebic dysentery or amoebiasis. By utilizing PVA-fixative it is possible to preserve these fragile organisms for subsequent examination. Suspected specimen material can be preserved and either forwarded to a central laboratory for confirmation or stained at a later time and trophozoites if present can be demonstrated. Other uses of this method include the preservation of material for recheck in cases diagnosed as positive as well as the preservation of good specimens for preparation of slides for teaching and reference purposes.

PVA-Fixative Technique (Goldman and Brooke):

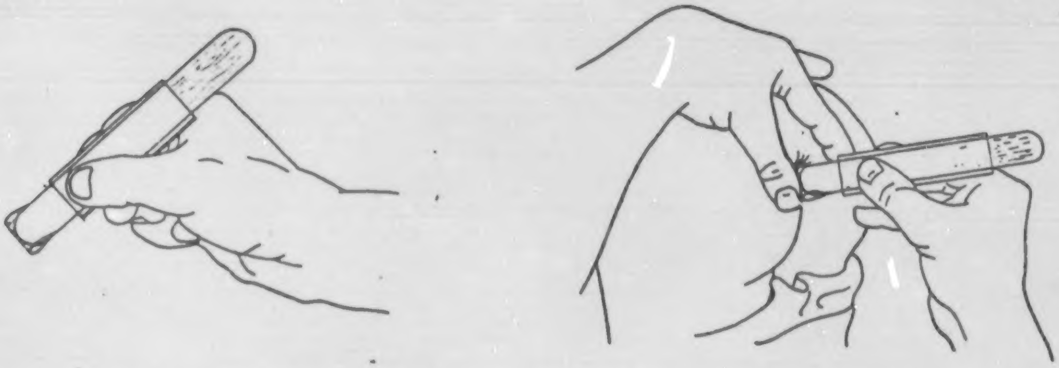
Materials and Equipments

Slides
Coverslips
Applicator sticks
Medicine dropper
PVA-fixative, prepared solution (56)



a. Hold slide against tongue depressor and lift long portion of tape from slide.

b. Loop tape over tongue depressor to expose gummed surface.



c. Hold tape against tongue depressor.

d. Touch gummed surface several times to perianal region.

Figure 1–1. Technique for Recovery of Pinworm Ova.

Technique:

1. It is most important to fix specimens immediately after passage from the patient before the organisms lose their characteristic morphology.
2. Fixation is accomplished by placing a drop of dysenteric stool or a small portion of normal stool on a slide with an applicator stick. With a medicine dropper add 3 drops of fixative.
3. Mix feces and fixative thoroughly with the applicator stick and smear the mixture over an area about an inch square in the center of the slide.
4. Place slides in slide boxes and cover to protect from dust. Allow slides to dry for several days at room temperature or overnight in a 37° C. incubator. Dried smears remain satisfactory for staining for many months.
5. PVA smears are permeable to all commonly employed staining reagents. They are handled in the same manner as smears fixed with Schaudinn's fixative. Staining utilizing the long Heidenhain iron-hematoxylin procedure gives the best results. Any of hematoxylin staining procedures described later in this section can be followed.

PVA-Fixative Technique (Ball). While the original PVA technique described above is excellent for trophozoites, cysts tend to collapse and also to stain unevenly when that method is followed. The technique described below preserves both cysts and trophozoites.

Materials and Equipment:

- PVA-fixative, prepared solution (56)
- Applicator sticks
- Slides
- Centrifuge tubes
- Pipette with rubber bulb attached
- Alcohol, ethyl, 95 percent
- Petri dishes
- Alcohol, 70 percent with 0.5 gm iodine added to each 100 ml alcohol

Technique:

1. Place a drop of PVA-alcohol fixative on the center of a slide. With an applicator stick spread the fixative evenly over an area about 22 x 22 mm. Dry the film in a 37° C. incubator overnight.
2. Fix specimen by mixing 3 parts of PVA-fixative solution to 1 part of feces. Specimens fixed in this manner can be either mounted immediately or kept indefinitely.
3. Preparatory to mounting the specimen (a mixture of 1 part feces and 3 parts PVA-fixative) place it in a centrifuge tube and centrifuge at 2,000 rpm for 5 minutes.
4. Decant the supernatant fluid. With a pipette with a rubber bulb attached withdraw a drop of the sediment and place it on the dried PVA-fixative film. With an applicator stick spread the specimen thinly and evenly over the dried film.
5. Place the slide face down in a Petri dish containing 95 percent alcohol for about 10 minutes. Support the slide on broken applicator sticks or toothpicks to prevent the smear from coming into contact with the bottom of the dish.
6. Transfer the slide to 70 percent iodized alcohol for 5 minutes. From this point proceed with the step following 70 percent iodized alcohol in either short or long iron-hematoxylin techniques.

PREPARATION AND EXAMINATION OF PERMANENT MOUNTS

The most satisfactory and most frequently employed permanent stain for intestinal protozoa is iron-hematoxylin. Various modifications of this staining technique have been developed. Fundamental steps in the various modifications include fixing, mordanting, staining, destaining, and dehydrating. For permanence of stain and definitive morphology the longer methods give the best results. Where permanence of stain is not too important a consideration, more rapid methods can be used to advantage.

Several points regarding the use of these procedures are stressed: The stool specimen should be freshly passed and must not be one obtained following purgation, administration of barium, or oil. The smear should be thin and made on a clean grease-free slide with a toothpick or applicator stick. At no stage in the entire process should the preparation be allowed to dry. Slides which dry at any stage in the procedure should be discarded. If the decolorizer is not thoroughly washed out of the specimen it will fade the stain. If dehydration is not complete, the xylol will become milky, and, when viewed microscopically after mounting, the preparation will appear blurred.

Iron-Hematoxylin Stains

Iron-Hematoxylin—Long Method:

Materials and Equipment:

- Coplin jars or staining dishes
- Slides
- Coverslips
- Applicator sticks
- Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)
- Alcohol, ethyl, 70 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)

Alcohol, ethyl, 50 percent
Iron alum, 4 percent solution
Hematoxylin, 0.5 percent solution
Iron alum, 2 percent solution
Alcohol, ethyl, 70 percent (plus a few drops saturated lithium carbonate)
Alcohol, ethyl, 95 percent
Alcohol, ethyl, absolute
Carbol-xylol (18)
Xylol
Balsam, clarite, or other mounting medium dissolved in xylol

Technique:

1. Prepare at least 5 smears on each specimen. Streak the feces on the center of the slide with an applicator stick spreading it over an area about the size of a quarter. Streaking should be lengthwise to the long axis of the slide since smears made in this manner are easier on the eyes of the examiner. Proper streaking also produces variations in thickness of the preparations resulting in intergrading differences in staining intensity.
2. Without allowing the specimen to dry place the slides immediately into Schaudinn's solution. The entire procedure can be carried out either in Coplin jars or staining dishes. Fixation can be hastened by heating the fixative to 50° C. and fixing for 5 minutes, or it can be carried out at room temperature for 1 hour.
3. Transfer slides to iodized 70 percent alcohol for 5 minutes. (The iodine combines with mercury to precipitate excess mercury as mercuric iodide.)
4. Transfer the slides to 50 percent alcohol for 3 minutes and then to tap water for 3 minutes.
5. Transfer slides to 4 percent iron alum mordant. Mordanting can be hastened by heating to 40°–50° C. for 10–20 minutes or the process can be carried out at room temperature by leaving slides in mordant solution overnight.
6. Place slides in 2 successive changes of tap water or distilled water to wash out mordant. Total time for these two changes should be about 3 minutes.
7. Stain slides by immersing for 5–10 minutes in 0.5 percent hematoxylin heated to 40°–50° C. or stain them overnight at room temperature.
8. Place slides in 2 successive changes of tap water or distilled water to wash out excess stain. Total time for these 2 changes should be about 3 minutes.
9. Destain to desired color intensity by transferring slides to 2 percent iron alum. This is one of the most critical steps in this technique. Factors influencing destaining time include the thickness of the smear, the organisms, and the reactions of the particular lots of reagents worked with. The process generally takes from 1–5 minutes. This step should be controlled by timing the destaining on the first slide processed; the destaining is observed microscopically. Time destaining on remaining slides accordingly.
10. Wash destaining solution out thoroughly utilizing several successive changes of distilled or tap water or placing the vessel containing the slides under running tap water. Wash from 5–30 minutes depending upon permanence of stain desired.
11. Transfer slides to 70 percent alcohol-lithium carbonate solution and leave for 3 minutes. Lithium carbonate intensifies the blueness of the stain.
12. Transfer to 95 and 100 percent alcohol for 3 minutes each and then to carbol-xylol for 5 minutes.
13. Clear in xylol for 3 minutes.

14. Mount in balsam, clarite, or other medium dissolved in xylol. About 3 drops of medium are adequate. Excessive amounts make slides difficult to work with due to the fact that excess medium runs out from beneath coverslips. For more rapid drying, slides can be placed horizontally in a 37° C. incubator.

Modified Heidenhain Stain (Diamond):

Materials and Equipment:

Coplin jars or staining dishes
Slides
Coverslips
Applicator sticks
Physiological saline (54)
Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)
Alcohol, ethyl, 50 percent
Iron alum, 4 percent solution
Tergitol-hematoxylin (68)
Picric acid, saturated aqueous solution
Alcohol, ethyl, 70 percent with potassium acetate added
Alcohol, ethyl, 95 percent
Alcohol, ethyl, absolute
Xylol
Balsam, clarite, or other mounting medium dissolved in xylol

Technique:

1. Emulsify a small bit of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or too viscous.
2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.
3. Fix immediately for 2 minutes in Schaudinn's fixative heated to 60° C.
4. Transfer to 50 percent alcohol for 1 minute and follow with a rinse in distilled water.
5. Mordant for 5 minutes in 4 percent iron alum, followed by a quick rinse in distilled water.
6. Stain for 5 minutes in tergitol-hematoxylin.
7. Rinse in distilled water and destain in a saturated aqueous solution of picric acid. This step will take 3–5 minutes depending upon the thickness of the smear.
8. Rinse in 2 changes of distilled water of about 30 seconds each.
9. Set stain by bluing in 70 percent alcohol to which has been added a few mg of potassium acetate to raise the pH above 7. Bromthymol blue or phenol red may be used as an indicator.
10. Pass successively through 70 percent alcohol, 1 minute; 95 percent alcohol, 1 minute; absolute alcohol, 2 minutes; and xylol, 2 changes of 1 minute each. Mount in any medium dissolved in xylol.

Modified Heidenhain Stain (Markey et al.):

Materials and Equipment:

Physiological saline (54)
Applicator sticks
Slides
Coverslips
Coplin jars or staining dishes
Schaudinn's fixative (5 ml acetic acid added to each 100 ml just before use) (63)
Alcohol, ethyl, 95 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)
Iron alum, 4 percent solution
Hematoxylin, 0.5 percent solution
Alcohol, ethyl, 95 percent
Alcohol, ethyl, absolute
Xylol
Balsalm, clarite, or any other medium dissolved in xylol

Technique:

1. Emulsify a small portion of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or too viscous.
2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.

3. Fix smears in Schaudinn's for 2 minutes or more.
4. Immerse in 95 percent iodized alcohol for 1 minute.
5. Rinse in tap water.
6. Mordant for 3 minutes at 56° C. in iron alum, and follow with a rinse in tap water.
7. Stain for 2 minutes at 56° C. in 0.5 percent iron hematoxylin.
8. Rinse in water and allow to stand until blue-black. To prevent fading slides should be washed in running water for 15–30 minutes.
9. Dehydrate in 95 percent alcohol for 1 minute, followed by absolute alcohol or acetone for 1 minute.
10. Clear in xylol and mount in any medium dissolved in xylol.

Modified Heidenhain Stain (Tomkins et al.):

Materials and Equipment:

Physiological saline (54)
Applicator sticks
Slides
Coverslips
Coplin jars or staining dishes
Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)
Alcohol, ethyl, 70 percent
Alcohol, 70 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)
Alcohol, ethyl, 50 percent
Iron alum, 4 percent solution
Hematoxylin, 0.5 percent solution
Phosphotungstic acid, 2 percent solution
Alcohol, ethyl, 70 percent, plus a few drops of saturated lithium carbonate
Alcohol, ethyl, 95 percent
Alcohol, ethyl, absolute
Carbol-xylol (18)
Xylol
Balsam, clarite or other mounting medium dissolved in xylol

Technique:

1. Emulsify a small portion of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or to vicous.
2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.
3. Fix smears in Schaudinn's solution for 5 minutes at 50° C. or fix for 1 hour at room temperature.
4. Transfer smears to iodized 70 percent alcohol for 5 minutes.
5. Move smears to 50 percent alcohol for 3 minutes.
6. Rinse smears in tap water.
7. Transfer smears to 4 percent iron alum mordant for 5 minutes.
8. Rinse smears 2 times in tap water.
9. Stain in 0.5 percent hematoxylin for 1 minute and rinse again in tap water.
10. Transfer preparations to 2 percent phosphotungstic acid destaining solution. Leave smears in this solution for a minimum of 2 minutes. With this method destaining is automatic and does not require control by microscopic observation.
11. Rinse smears in running tap water for 5 minutes.
12. Transfer smears to 70 percent alcohol-lithium carbonate solution and leave for 3 minutes.
13. Transfer smears successively to 95 and 100 percent alcohol for 3 minutes each, carbol-xylol for 5 minutes and xylol for 5 minutes.
14. Mount preparations in any medium dissolved in xylol.

Tannic Acid Fixative Method for Staining Protozoa (Alli):

Materials and Equipment:

Applicator sticks
Slides
Coverslips
Petri dishes
Tannic acid fixative (67)
Iron alum, 4 percent solution
Hematoxylin, 0.5 percent solution
Decolorizer (1 percent solution of acetic acid in 70 percent alcohol)
Alcohol, ethyl, 50 percent

Alcohol, ethyl, 70 percent (with trace of potassium acetate added)
Alcohol, ethyl, 80 percent
Alcohol, ethyl, 95 percent
Alcohol, ethyl, absolute
Carbol-xylol (18)
Balsalm, clarite or any other mounting medium dissolved in xylol

Technique:

1. With a wooden applicator stick make 6–12 smears on standard sized round or square coverslips. Handle coverslips with forceps in all steps in this procedure. Drop coverslips face down in a Petri dish one-half filled with tannic acid fixative. (Entire procedure is carried out in Petri dishes.) Let stand 5–7 minutes.
2. Transfer coverslips face down to Petri dish containing tap water. Rinse thoroughly by changing tap water 3 or 4 times.
3. Transfer coverslips to iron alum mordant solution turning them face up as they are transferred. Let stand for 5 minutes. The mordant solution is discarded after each use.
4. Transfer coverslips face up to Petri dish containing tap water. Rinse thoroughly by changing water 3 or 4 times.
5. Transfer coverslips to Heidenhain's hematoxylin face up.
6. Transfer coverslips to Petri dish containing tap water. Rinse thoroughly by changing water 2 or 3 times.
7. Transfer coverslips face up to decoloring solution and allow to decolorize from 30 seconds to 1 minute (never longer than 3 minutes) until desired blue color appears.
8. Pass coverslip face up through 2 changes of 50 percent alcohol, then to 70 percent alcohol with a trace of potassium acetate added (to intensify blueness) and on through 80 percent and 95 percent alcohol. Leave the preparation in each for about 30 seconds.
9. Pass the preparations face up to absolute alcohol and leave for several minutes.
10. Pass through 2 dishes containing carbol-xylol leaving cover slips in each dish about 3 minutes. Mount in any medium dissolved in xylol by turning coverslips face down onto 2 or 3 drops of medium on a slide.

Concentration and Permanent Staining of PVA-Preserved Fecal Specimens:

Materials and Equipment:

Beaker, 50 ml
Applicator sticks
Funnel, 50 ml
Gauze
Ether
Centrifuge tube, with cork
Cleansing tissue
Slides
Coverslips
Iodine stain or iodized alcohol, 70 percent (see steps 7 and 8 below)

Technique:

1. With applicator sticks emulsify 1–2 grams of the preserved fecal specimen in 10 ml of tap water in a 50 ml beaker.

Note: Specimens are preserved by mixing 1 part of feces to 3 parts PVA solution.

2. Line a 50 ml funnel with 1 thickness of gauze and filter the preparation.
3. Transfer the filtrate to a centrifuge tube and add 1 ml of ether to the suspension, cork it and shake it vigorously.
4. Centrifuge the preparation for 3–5 minutes at 2,400 rpm. With an applicator stick ring the plug of detritus and discard the supernate and plug retaining only the sediment in the bottom of the centrifuge tube.
5. Transfer the sediment to a piece of cleansing tissue to absorb the excess PVA. Leave the sediment on

the cleansing tissue for a period of 3-5 minutes.

6. Smear the moist residue on a slide with an applicator stick.
7. To make a direct examination mix the concentrated fecal specimen with a drop of temporary iodine stain, coverslip and examine.
8. To prepare a permanent mount place the smear in iodized alcohol, 70 percent and proceed from this step following any of the hematoxylin staining procedures or the trichrome staining procedure described elsewhere in this manual.

Phosphotungstic Acid Hematoxylin Technique for Staining Intestinal Protozoa:

Materials and Equipment:

Applicator sticks
Slides
Coverslips
Coplin jars
Schaudinn's fixative (63)
Alcohol, ethyl, 30, 50, and 70 percent
Alcohol, ethyl, absolute
Alcohol, ethyl, 70 percent, iodized
Sodium hyposulfite solution (66)
Hematoxylin stain (36)
Xylol
Balsalm, synthetic resin or other suitable medium

Technique:

1. Emulsify a bit of feces about the size of a pea in physiological saline. Avoid making the emulsion too fluid or to viscous.
2. With an applicator stick spread a thin film of the emulsion on a chemically clean glass slide.
3. Fix in Schaudinn's fixative for 30 minutes.
4. Pass slides to 70 percent ethyl alcohol for 10 minutes.
5. Transfer slides to sodium hyposulfite solution for 10 minutes.
6. Remove sodium hyposulfite by immersing slides in two changes of 70 percent ethyl alcohol, 2 minutes each (manually agitate slides in this solution occasionally during this time interval).
7. Immerse slides in absolute ethyl alcohol for 10 minutes to harden parasites.
8. Transfer slides consecutively to 70, 50, and 30 percent ethyl alcohol, 3 minutes each.
9. Place in hematoxylin stain for 12 hours or overnight at room temperature or in a 37° C. incubator for 4 hours.
10. Wash in tap water for 1 minute.
11. Dehydrate rapidly by successive transfer of slides to 30, 50, and 70 percent alcohol for 1 minute each.
12. Pass slides through 2 successive changes of absolute ethyl alcohol, 3 minutes each.
13. Clear in 2 successive changes of xylol for 5 minutes each.
14. Cover mount with 2 or 3 drops of synthetic resin or Canada balsam, and coverslip preparations.

Acid Fuchsin Fast Green Staining of Intestinal Protozoa (Lawless).

Materials and Equipment:

Applicator sticks
Slides
Coverslips
Medicine dropper
Acid Fuchsin-fast green fixative (2)
Alcohol, ethyl, 50, 70, 95 percent
Alcohol, ethyl, absolute
Xylol
Balsam, damar, or other suitable medium dissolved in xylol

Technique:

1. Using an applicator stick, smear a thin layer of feces on the slide. At *no time* in the procedure should the smear be allowed to dry.
2. Using a medicine dropper, immediately cover the preparation with the fixative-stain, making sure the preparation is completely covered.
3. Gently heat the stain covered smear over a burner or alcohol lamp until steam is first observed. Two or three passes of the flame are usually

sufficient. *Do not boil or allow the solution to flame.*

4. Wash immediately in tap water.
5. Place in 50 and 70 percent alcohol for 30 seconds each.
6. Transfer slides to 95 percent and absolute alcohol for 15 seconds each.
7. Clear the preparations in xylol for 1 minute or more.
8. Mount in balsam, damar, or other suitable medium dissolved in xylol.

Preparation of Smears from Zinc Sulfate Concentrated Stools for Trichrome or Hematoxylin Staining:

Materials and Equipment:

Wire loop
Human serum
Slides
Coverslips
Schaudinn's fixative solution (63)

Technique:

1. Prepare zinc sulfate concentrated stool according to method described elsewhere in this manual.
2. Allow several minutes after concentration of the stool to permit time for parasites to float to the surface.
3. With a wire loop rapidly apply a loopful of human serum to the surface film of the concentrated stool.
4. After allowing time for the serum to spread over the meniscus touch a wire loop approximately 5 mm in diameter to the surface film, removing several loopfuls in this manner to a glass slide. It is important that the loop be parallel to the surface film and the holder held perpendicular to the film.
5. The serum and adherent material is smeared with a rotational movement over the glass slide.
6. Fix preparations in Schaudinn's fixative for 30 minutes.
7. Stain with either hematoxylin or trichrome stain following any of the methods described in this manual. (Proceed with step following fixation.)

Note: **Formalized, ether extracted, and PVA-preserved stools do not lend themselves to this method.**

The Preparation and Trichrome Staining of PVA-Preserved Specimens or Schaudinn's Fixed Fresh Stools:

Materials and Equipment:

Slides
Coverslips
Alcohol, ethyl, iodized, 70 percent
Alcohol, ethyl, 70 percent
Trichrome stain (69)
Alcohol, ethyl, 90 percent, acidified (Acidify by adding 1 drop of glacial acetic acid to each 10 ml of alcohol)
Alcohol, ethyl, absolute
Xylol
Xylo-damar, balsam, permount or other suitable mounting medium

Technique:

1. Place PVA or Schaudinn's fixed preparations in 70 percent iodized ethyl alcohol for 1 minute. (See technique for preparation of PVA smears described elsewhere in this manual.)
2. Pass specimens to 70 percent ethyl alcohol for 1 minute.
3. Pass specimens through a second change of 70 percent ethyl alcohol for 1 minute.
4. Place specimens in trichrome stain for 5–10 minutes. The shorter period of time is satisfactory if stools are very soft or liquid.
5. Pass preparations to 90 percent acidified ethyl alcohol for 10–20 seconds or until the stain barely runs from the smear.
6. Rinse slides in 2 changes of absolute ethyl alcohol.
7. Pass slides to fresh absolute ethyl alcohol and leave for 1 minute.
8. Clear preparations in xylol for 1 minute or longer. The slides should not be removed from the xylol until the preparations are clear.
9. Add 2 or 3 drops of permount or other suitable mounting medium and coverslip.

THE CULTURE OF INTESTINAL PROTOZOANS

A number of different media have been developed for the culture of intestinal protozoa. Claims to the effect that one or the other of these media is superior to the other are probably unfounded. Different degrees of success with the different types probably represent differences in the amount of experience acquired in working with them. Their value as diagnostic procedures has been questioned by many workers. There are some who point out that culture techniques are most time-consuming and that an equivalent amount of time spent in examination of a larger number of stools on each patient and a more thorough search of each specimen will serve to detect a larger number of positives. A technician's actual experience in routine follow-up of specimens found negative by other techniques will serve to indicate whether routine culture of such specimens is worthwhile. Three of the more commonly used media will be outlined here.

For the benefit of persons who have had no prior experience with protozoan culture several points are stressed. With the exception of *Giardia lamblia* and *Balantidium coli* all human intestinal protozoa can be cultured. There are differences in culturability of different strains of protozoa within the same species. Large strains of *Endamoeba histolytica*, for example, are more readily cultured than the smaller strains. Some strains cannot be cultured at all while others are very readily cultured. Trophozoites, especially those of amoebae, tend to be atypical in culture, presenting nuclear morphology quite different from that seen in trophozoites in stools. Different protozoa are more readily recovered from different levels in the media. Amoebae in general will be found at the very bottom of the fluid, *Chilomastix mesnili* is found most abundantly at the middle level, while *Trichomonas hominis* occurs in greatest abundance near the upper surface. Negative findings in cultures cannot be accepted as an indication that a patient is free from infection.

In examining protozoan cultures for organisms it is most important that the mounted preparation be kept warm during examination. The slide should be warmed in the incubator to 37° C. before the specimen is transferred thereto. The examination should be performed in a warm room. After the mount is prepared it should be returned to the incubator for about 5 minutes to allow time for the organisms to commence normal locomotion.

Protozoan cultures are often maintained in laboratories for teaching purposes. Such cultures must be transplanted three times weekly and are generally serially transferred on Mondays, Wednesdays, and Fridays. The quantity of inoculum utilized is about 0.5 ml, and location in the culture from which the inoculum is taken must take into account the growth habits of the particular species involved. To assure against loss of cultures, the old cultures are retained until a subsequent transfer is made from the new ones. The cultures should be checked each time prior to transfer. Maintenance of such cultures in the laboratory is not easy and offers a real challenge to the technician.

Principal sources of difficulty in the culture of protozoans are:

1. *In the preparation of the medium:*
    a. Fresh eggs (not more than 1 day old) should be used. A "blood spot" in a fresh egg is not harmful.
    b. Chemically clean glassware should be used exclusively. Wherever possible all glassware and equipment used in preparation of medium should be sterile. This will prevent excessive contamination which often cannot be effectively eliminated by the maximal sterilization which the medium will tolerate.
    c. If an autoclave must be used for inspissating medium, temperature and pressure must be most carefully controlled or the medium may bubble, be scorched, or inadequately sterilized. Commercial inspissators are much more satisfactory if available.
    d. The pH is critical and must be maintained at the specified level.

e. Medium should be checked for sterility, refrigerated, and used within three weeks after preparation.

2. *In inoculation and transfer:*
   a. Wherever possible inoculation and transfer of cultures should be performed in an area where there is the least chance of aerial contamination.
   b. Cultures should not be allowed to stand at room temperature any longer than necessary.
   c. In transferring cultures the inoculum must be taken from the proper depth.
   d. When cultures are being maintained the time interval between transfers should not exceed 72 hours.
3. *In identifying the species from culture material:*
   a. Trophic forms are frequently so atypical both in morphology and motility that they cannot be differentiated on direct examination.
   b. Cultures in which trophic forms are found should be incubated for an additional 24 hours and examined for cysts. The latter display characteristic morphology and permit accurate identification.

Media for Culture of Protozoans

Boeck and Drbohlav's Medium. *Modified Locke's Solution for Boeck and Drbohlav's Medium:*

Materials and Equipment:

Distilled water	1,000.00 ml
Sodium chloride	8.00 gm
Calcium chloride	0.20 gm
Potassium chloride	2.20 gm
Magnesium chloride	0.01 gm
Disodium hydrogen phosphate	2.00 gm
Sodium bicarbonate	0.40 gm
Potassium dihydrogen phosphate	0.30 gm
Flask, Erlenmeyer	2000 ml
Funnel	150 ml
Filter paper	

Technique:

1. Add chemicals to the distilled water in the order listed. After adding each chemical mix thoroughly until dissolved. Heat for 20 minutes.
2. A precipitate will form. Cool to room temperature and filter through paper.
3. Dispense in suitable sized containers and sterilize in autoclave for 15 minutes at 15 pounds of pressure. Final pH should be 7.5.

Preparation of Slants:

Materials and Equipment:

Fresh eggs	4
Sterile Locke's solution	50 ml
Alcohol, ethyl	70 percent
Flask	250 ml
Glass beads	
Funnel	50 ml
Gauze	
Test tubes	15 ml
Cotton, absorbent	

Technique:

1. Wash 4 fresh eggs with soap and water, rinse in 70 percent alcohol.
2. Break the eggs into a flask with beads. (A Waring blender should be used if available.)
3. Add the sterile Locke's solution and emulsify.
4. Filter the mixture through gauze and dispense 5 ml quantities of the medium into 15 ml test tubes and plug the tubes with absorbent cotton.
5. Inspissate in the autoclave.
6. Place the tubes in the chamber in the desired slanted position. If the autoclave has a jacket, allow the pressure to reach 15 pounds. Close all exhaust valves and the door of the chamber. This procedure will entrap all the air in the chamber. Allow the steam from the jacket to enter the chamber as fast as possible until pressure gauge registers 15 pounds. Hold at this pressure for 15 minutes. Cut off steam supply and allow pressure to drop

gradually with all doors and valves tightly closed.

7. If the autoclave has no jacket, place the tubes in the chamber, close all exhaust valves and the door tightly. (If the drain at the bottom of the autoclave has no valve, it may be stoppered with a rubber stopper.) Turn on the steam and allow the pressure to rise to 15 pounds. (The temperature of the mixture of air and steam will not be above 85° C.) Hold at this pressure for 15 minutes Turn off steam and allow autoclave to cool gradually. Sudden drops of pressure tend to disrupt the medium causing bubbles and/or rough surfaces.
8. When the inspissated slants are cold, cover them to a depth of 1 cm with sterile Locke's solution. Sterilize in the autoclave at 15 pounds pressure for 15 minutes. Allow pressure to drop very gradually. Test for sterility by incubating at 37° C. for 24 hours, and store tubes in refrigerator until needed.

Note: Just prior to use add 2 or 3 loopfuls of sterile rice flour to fluid overlay in each tube.

Modified Cleveland and Sanders Medium for Endamoeba Histolytica.

Serum-saline Solution:

Materials and Equipment:

$Na_2HPO_4.12H_2O$	11.23 gm
KH_2PO_4	0.26 gm
NaCl	8.00 gm
Distilled water	1,000.00 ml
Sterile horse serum	100.00 ml
Flask, Erlenmeyer	2000.00 ml

Technique:

1. Dissolve the three salts in the distilled water and autoclave this solution at 15 pounds pressure for 20 minutes.
2. Cool and add the sterile horse serum. Add sufficient serum-saline solution to each slant to cover about three-fourths of the slant.

Base Medium:

Materials and Equipment:

Entamoeba medium, dehydrated (Difco)	33 gm
Distilled water	1,000 ml
Flask, Erlenmeyer	2000 ml
Test tubes	15 ml
Cotton, absorbent	

Technique:

1. Dissolve the dehydrated medium in the distilled water, dispense the medium in 5 ml quantities in 15 ml test tubes, plug with absorbent cotton, and autoclave at 15 pounds pressure for 20 minutes.
2. Slant and leave at room temperature for several days until the slants are hardened.
3. Incubate overnight at 37° C. to test for sterility. Store in refrigerator.

Note: Just prior to use add 2 or 3 loopfuls of sterile rice flour to fluid overlay in each tube.

BALAMUTH'S EGG INFUSION MEDIUM.

Materials and Equipment:

NaCl solution 0.8 percent	125 ml
Wilson's dry extract of liver (powder)	5 gm
Distilled water	120 ml
Boiled yolks of fresh eggs (boil 15 minutes)	4
M/15 Potassium phosphate solution buffered to pH 7.5	
Waring blender	
Double boiler with cover	
Buchner funnel	
Filter paper	#2
Test tubes	15 ml
Cotton, absorbent	

Technique:

1. Mix the egg yolks with the 0.8 percent NaCl in a Waring blender.
2. Heat the mixture in a covered double boiler. When the temperature reaches 80° C. add 20 ml of the distilled water to make up for evaporation.
3. Filter the mixture through a Buchner funnel under reduced pressure using several layers of #2 filter

paper. Autoclave the filtrate for 20 minutes at 15 pounds pressure. Cool to below 10° C. and refilter through Buchner funnel. Measure the filtrate and add an equal quantity of M/15 potassium phosphate solution.

4. To 100 ml of distilled water add 5 gm of dry extract of liver and bring it to a boil.
5. Filter and autoclave the filtrate at 15 pounds pressure for 20 minutes.
6. The two solutions are now mixed in the ratio of 1 part of liver extract solution to 9 parts of the egg yolk infusion.
7. Autoclave again at 15 pounds pressure for 20 minutes.
8. Tube in sterile test tubes in 5 ml amounts, plug the tubes with absorbent cotton, incubate medium for 24 hours at 37° C., and store in the refrigerator.

Note: Two or 3 loopfuls of sterile rice starch are added to the medium just before use.

Inoculation and Examination of Cultures for Intestinal Protozoans:

Materials and Equipment:

- Boeck and Drbohlav's, Balamuth's, or Cleveland and Sanders' medium
- Sterile rice powder
- Platinum loop
- Penicillin and Streptomycin solution (5000 units of each per ml)
- Pipette, large bore, with attached rubber bulb
- Applicator sticks

Technique:

1. Prior to inoculation tubed medium is placed in a 37° C. incubator for a few hours to bring the medium up to incubating temperature.
2. Add 2 or 3 loopfuls of sterile rice flour to each tube of medium just prior to use. (Rice flour is sterilized by dispensing it in approximately 1 gm quantities in Wassermann tubes and heating in a dry oven for 2½ hours at 150° C. The flour can be conveniently dispensed with a sterile platinum loop. Use a fresh tube of rice flour each time a batch of tubes is inoculated.)
3. Inoculate 2 tubes from each specimen. One of the tubes is treated with antibiotics at the rate of 250 units each of penicillin and streptomycin per ml of fluid in the tube. (5,000 units of each per 1 ml. Therefore, 250 units of each will be contained in .05 ml or approximately 1 drop.)
4. If the stool is fluid, transfer 0.5 ml of the specimen to each tube with a pipette with attached rubber bulb.
5. To inoculate formed stools select a portion of stool about the size of a pea, taking material from several parts of the specimen with an applicator stick. Transfer the feces to the fluid medium and macerate it against the side of the tube to mix it thoroughly.
6. Incubate tubes at 37° C. After 24 hours of incubation check for growth by removing a few drops from the bottom of the tube if utilizing a fluid medium, or from the base of the slant if utilizing a fluid overlay over a hard base medium.
7. If negative recheck the specimen after 48 hours of incubation. Specimens negative after 48 hours can either be discarded or retained for an additional 24 hours and examined once more. Retention of specimens for 72 hours enhance cyst formation.
8. For definitive diagnosis positive specimens can be processed by either of the PVA-fixative techniques discussed elsewhere in this manual.

RECOMMENDED ROUTINE FOR STOOL SPECIMENS

1. Careful and thorough macroscopic examination.
2. Three direct wet smears with saline and iodine-stained halves. (D'Antoni's Iodine solution recommended.)
3. A zinc sulfate flotation preparation. (Faust's method recommended.)
4. Permanent iron-hematoxylin stained slide on all positive specimens for definitive species diagnosis (protozoan cysts and trophozoites) and permanent file record.
5. Acid-ether preparation on cases suspected of harboring operculate or Schistosome ova but not confirmed by routine wet smears. (Telemann's method recommended.)

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Chapter 2

THE PREPARATION OF PERMANENT MOUNTS OF HELMINTHS

The preparation of permanent mounts of helminths is a laboratory procedure which is quite frequently performed, particularly in large overseas hospitals where helminth infections are frequently encountered. The two principal reasons for making such preparations are for the laboratory diagnosis of helminth infections based on observation of the morphology of adults recovered in stool examinations, and secondly for the preparation of materials for reference as well as for use in on-the-job training of laboratory personnel.

PREPARATION OF HELMINTHS FOR STAINING

Removal of Helminths From the Host

For preparation of good whole mounts the specimens should, whenever possible, be obtained in living condition. The most suitable material for good mounts is obtained by dissecting freshly killed host animals. Since a large majority of helminths will be found in the intestines, the proper method for removing worms from the alimentary tract will be briefly described.

First, expose the entire viscera. If the host animal is relatively small, tie the gut off with string at the esophageal and rectal ends. Grasp the rectal end with a forceps and with sharp shears carefully cut away the connecting mesenteries and other tissues to free the gut throughout its length. For larger animals tie off and cut out shorter portions thereof and transfer to a dish of physiological saline and with a fine shears start at one end and slit the intestine open lengthwise to expose the lumen. With a forceps agitate the gut gently in the fluid. This will dislodge the free and less securely attached organisms. Forms securely attached by suckers or hooks should not be forcibly dislodged. The latter may be caused to free themselves from attachment by placing the material in the refrigerator for a few hours. Dissecting away the tissue around points of attachment and occasional agitation of the dish will hasten the process. When dealing with smaller species, the entire contents of the dish should be carefully examined under a dissecting microscope.

To prevent rupture and distortion of freed organisms, transfer them with blunt instruments. Very small organisms can be aspirated and transferred to fresh physiological saline with a wide mouth pipette. If large enough, the worms may be transferred with camel's-hair brush, small spatula, or wire loop. Glass rods or any suitable blunt instrument can be used to handle the larger helminths since they are less fragile.

Helminths inhabiting tissues, such as liver flukes, lung flukes, and cysticerci must be very carefully dissected out. Liver flukes, for example, are the same coloration as the tissue in which they occur and must be carefully searched for. The entire organ is first cut into pieces of approximately one pound each. Each piece is dissected by tearing along the course of the larger ducts, and the worms are squeezed out.

Cleansing of Helminths

Following removal from the host, the worms should be transferred carefully through several changes of saline and gently agitated in each change to remove all adhering mucus and debris. A soft brush can be used to remove particles that cannot be loosened by shaking the dish, the latter being best accomplished under a binocular dissecting scope. If specimens are plentiful, the damaged ones should be discarded at this point.

Relaxation of Living Helminths

Various methods may be used to cause helminths to relax to preserve normal morphology. The technique of choice depends chiefly upon the size and the particular parasite with which one may be dealing.

Very small trematodes can be relaxed by placing them in several drops of saline on a glass slide, coverslipping, exerting gentle pressure on the coverslip, and then cooling in the refrigerator for about an hour. Larger trematodes may be placed in a wide mouth jar half filled with saline and the contents shaken vigorously for several minutes to cause the worms to relax. Large trematodes may also be relaxed by placing them individually or in groups, depending upon size, on large glass slides and a few drops of fixative are then dropped on them. They are then quickly covered with a second glass slide and pressure is immediately applied to flatten them out to normal contour. Tie the slides securely together with string and submerge the entire preparation in the fixative. To prevent distortion, do not tie the slides together too tightly. Separate the slides slightly from time to time and force fixative between them to make certain that all parts of the organism are in contact with the fixative. Cestodes and trematodes may also be relaxed by placing them in physiological saline and leaving them for several hours or overnight in the refrigerator. Some trematodes, particularly schistosomes, tend to deteriorate if retained as long as 10 or 12 hours prior to fixation and these should, therefore, be relaxed for periods not exceeding about 3 hours prior to fixation. The larger tapeworms may be extended to normal length by wrapping them about strips of plate glass or around a large test tube or glass jar. Larger cestodes, the mounts of which will be individual proglottids or short chains of proglottids, can be relaxed by cutting in strips and compressing between two glass slides in the manner previously described for large trematodes. To prevent distortion or loss of the heads of tapeworms, they should be cut off first and handled individually throughout all procedures preparatory to mounting. Long strips of tapeworm proglottids can be cut from the stobila and placed alongside one another on pieces of heavy paper toweling which has been soaked in fixative. Gently flatten the strips and extend them to normal length. After the worms have been properly extended a piece of fixative-soaked paper toweling can be placed on top of them. After about 30 minutes sufficient additional fixative to cover, but not float, the paper should be added and fixation continued overnight.

Nematodes require no special handling to assure fully extended and relaxed specimens. Following cleansing, one may proceed to fixation without any intermediate steps.

Fixation

The proper fixation of helminths is absolutely esesntial for permanent preservation of the specimens. Fixation stops metabolic processes quickly and preserves actual form if accomplished when the organisms have been properly relaxed. It hardens the tissues, prevents regressive changes from taking place, and preserves the cytological and histological elements in their natural state. Numerous fixation techniques can be used. The simpler and more common ones will be described here.

Formalin fixation is rapid, simple, and inexpensive. A 5 percent steaming (not boiling) solution of formalin is recommended. Most of the saline is first poured off and the formalin solution is rapidly poured over the extended worms. After several hours, pour off the formalin and add fresh unheated 5 percent formalin. Since formalin is an excellent preservative as well as a fixing agent, the organisms can be left in it indefinitely provided the container is sealed to prevent evaporation. Prior to proceeding with any permanent mounting technique, the formalin must be washed out of the specimens by running tap water over them for several hours. This is easily accomplished by removing the top of the container and substituting several layers of cheesecloth secured in position by string or rubber band. The container is then placed under a cold water tap and the specimens are washed with slowly running water for several hours.

Other commonly used fixatives which will give more delicate fixation include alcohol-formalin-acetic acid fixative (A.F.A.), Gilson's mixture, and Schaudinn's fluid. The latter two fixatives contain mercury, and specimens must not be handled in them with metal instruments. Only glass rods or wooden sticks should be brought into contact with these fixatives. A.F.A. fixative should be used cold while Gilson's and Schaudinn's may be used either cold or as steaming solutions. If hot solutions are used, the organisms are obtained in a more natural and extended position. At some stage in the processing prior to mounting, specimens fixed in solutions containing mercury must be treated with iodized alcohol to precipitate the mercury. If A.F.A. fixative is used, allow the specimens to remain in the solution overnight and then transfer them directly to 70 percent alcohol in which they can be left indefinitely. Following fixation with Gilson's or Schaudinn's, transfer the specimens directly to 70 percent iodized alcohol and allow them to remain there with occasional agitation for 12–24 hours depending on the thickness of the organisms. The specimens are next transferred to 70 percent alcohol where they can be left indefinitely. It is stressed at this point that regardless of what fixative is used, specimens will make better mounts if they are processed through to a point where they can be stored in 70 percent alcohol following fixation.

The cuticula of nematodes is quite impervious, and the routine fixation methods discussed above are, therefore, not recommended for fixation. One commonly used technique is to place the living specimens in a small beaker and pour a considerable volume of hot glycerin-alcohol over them. They can be left in the glycerin-alcohol fixative indefinitely provided the container is sealed to prevent evaporation.

STAINING AND MOUNTING OF HELMINTHS AND HELMINTH OVA

Staining and mounting techniques will vary depending upon size of specimens, species, and the stage of development of the organisms. Various techniques applicable to processing of material, taking these factors into consideration, are set forth in this part. All procedures are written on the assumption that specimens were treated with iodine (if fixed in solutions containing mercury) to precipitate the mercury and then stored in 70 percent alcohol.

Staining and Mounting of Adult Trematodes

Large and Medium-Sized Specimens:

Materials and Equipment:

Alcohol, ethyl, 30, 35, 50, 70, 85 and 90 percent
Distilled water
Staining dishes
Stain (any stain listed in step 2)
Alcohol, ethyl, acidified (add 6 drops of conc. HCl to 100 ml of 35 percent alcohol)
Ammonia water, dilute (add 1 drop of conc. ammonia to 250 ml of distilled water)
Beechwood creosote or aniline oil
Xylol
Xylo-damar or balsam (73)
Slides
Coverslips
Applicator stick with spatulate end
Pipette, wide-mouthed, with attached rubber bulb

Technique:

1. Pass the specimens successively through 50 percent and 30 percent alcohol, 1 hour each and wash thoroughly in distilled water.
2. Stain overnight. (Use Mayer's paracarmine, Grenacher's borax carmine, Delafield's hematoxylin Ehrlich's acid hematoxylin, or Roudabush's hematin.)
3. Pass through three successive changes of tap water, 5 minutes each.

4. Destain in acidified 35 percent alcohol. With the carmine stains, destain until the specimens display a delicate pink color. With the hematoxylin stains, destain until the specimens are a light reddish-purple. This is a critical step and should be done under observation.
5. Transfer specimens to ammonia water for 30 minutes.
6. Pass through three successive changes of tap water, 5 minutes each.
7. Dehydrate by passing successively through 35, 50, 70, 85, and 90 percent ethyl alcohol, 30 minutes each.
8. Clear in beechwood creosote or aniline oil for 1 hour.
9. Wash in xylol, two changes, 5 minutes each.
10. Mount in xylo-damar or balsam. The mount is prepared by first transferring a few drops of fairly thick mounting medium to the center of a clean, grease-free slide. Transfer large specimens to the mounting medium on the slide with an applicator stick, the end of which has been cut to form a flat spatulate surface. Smaller specimens can be handled with a wide-mouthed pipette with attached rubber bulb. In transferring specimens from clearing agent to mounting medium, transfer as little xylol as possible to the medium. With the applicator stick, gently press the specimen down into the mounting medium. Care must be taken not to allow specimens to dry at any stage in the process. Additional medium is then added, the amount depending upon size and thickness of the preparation. Cover by bringing one edge of the coverslip into contact with the medium and drop it gradually with a rolling motion to prevent entrapment of bubbles. In mounting large specimens, bits of broken glass can be placed under the margins of the coverslip to prevent it from tipping. Dry in flat position for several weeks. Drying in a 37° C. incubator hastens the process.
11. If specimens turn opaque or white when they are placed in the medium, this is an indication that dehydration was incomplete. Such specimens can be returned to xylol until they are clear and then remounted. Specimens persistently opaquing can usually be cleared by placing them in xylol containing mounting medium. The quantity of medium in xylol is increased gradually. The container is left unstoppered to allow the xylol to evaporate. Specimens mounted in balsam have less tendency to become opaque than specimens mounted in damar.

Very Small Species of Adult and Larval Trematodes:

Materials and Equipment:

Alcohol, ethyl, 70, 80, 90 percent and absolute
Staining dishes, watch glasses, or evaporating dishes
Pipette with attached rubber bulb
Mayer's paracarmine stain (50)
Alcohol, ethyl, acidified (add 1 ml of conc. HCl to 100 ml of 70 percent alcohol)
Slides
Coverslips
Xylo-damar or balsam (73)

Technique:

1. Transfer specimens from the 70 percent alcohol preservative to a small dish or watch glass. The material is very conveniently processed in small round-bottomed porcelain evaporating dishes.
2. Allow time for specimens to settle to the bottom of the container, then carefully pipette off the alcohol leaving only a sufficient

amount in the bottom of the dish to prevent specimens from drying.

3. If working with very small specimens, check under a binocular dissecting scope to ascertain their presence before proceeding further.
4. Barely cover the specimens with Mayer's paracarmine and stain for a minimum of 1 hour.
5. To dilute the stain and minimize danger of loss of small specimens, fill the container with 70 percent alcohol. Allow time for the specimens to settle to the bottom and then pipette off excess alcohol. Fill the container again with 70 percent alcohol and withdraw alcohol by pipetting, repeating until the fluid is colorless. Retain and examine alcohol removed to make certain that no specimens are discarded.
6. Cover specimens with acidified 70 percent alcohol and destain under observation until specimens are a delicate pink color. Small specimens destain rapidly and must be observed closely to prevent complete bleaching of the stain. Generally, cestodes and small trematodes destain quite rapidly, usually requiring 10–30 minutes, depending on the size. The larger trematodes may take up to several hours to destain.
7. Pipette off as much of the acid alcohol as possible and cover specimens with 80 percent alcohol. Again remove as much of the alcohol as possible and cover with a fresh change of 80 percent alcohol leaving in this solution for a minimum of 30 minutes. If the alcohol assumes a pinkish hue, this step should be repeated to remove any traces of acid which may be present.
8. Pass successively through two changes of 95 percent alcohol, leaving in each change for 5 minutes or more.
9. Transfer specimens to absolute alcohol, leaving 5 minutes or more.
10. Pass specimens to second change of absolute alcohol, vaseline-seal the container, and let stand overnight. (*Note:* All changes are made by pipetting off as much fluid as possible.)
11. Remove excess alcohol and pass specimens through two successive changes of xylol of 5 minutes each.
12. Mount in thin xylo-damar or balsam following method previously described for adult trematodes.

Staining and Mounting of Cestodes

Delafield's Iron-Hematoxylin Stain:

Materials and Equipment:

Alcohol, ethyl, 30, 50, 70, 80, and 95 percent
Delafield's hematoxylin stain (21)
Distilled water
Staining dishes
Alcohol, ethyl, acidified (add 1 ml of conc. HCl to 100 ml of 70 percent alcohol)
Alcohol, ethyl, ammoniated (add 1 drop of conc. ammonia to 250 ml of 70 percent alcohol)
Beechwood creosote or aniline oil.
Xylol
Xylo-damar or balsam (73)
Slides
Coverslips

Technique:

1. Transfer specimens (stored in 70 percent alcohol) successively through 50 and 30 percent alcohol for 1 hour each.
2. Dilute stock Delafield's iron-hematoxylin 1–9 with distilled water just prior to use. Transfer specimens to staining solution and stain for several hours or overnight.
3. Wash in several changes of water to remove adhering stain.
4. Pass through successive changes of 30, 50, and 70 percent alcohol for

a minimum of 30 minutes each. Larger specimens should be left longer in each successive change.

5. Destain in 70 percent acidified alcohol until the specimens assume a delicate light reddish-purple color. If the alcohol becomes deeply colored, it should be changed.
6. Wash for a few minutes in 70 percent alcohol. Remove specimens to fresh 70 percent alcohol and leave for several hours. If alcohol takes on any coloration, this step should be repeated.
7. Place specimens in ammoniated 70 percent alcohol until they become bluish-purple.
8. Dehydrate successively in 80 and 95 percent alcohol for a minimum of 30 minutes each.
9. Clear in beechwood creosote or aniline oil for 1 hour. Wash in xylol, two changes 10 minutes each.
10. Mount in xylo-damar or balsam following method previously described for adult trematodes.

Semichon's Carmine Stain:

Materials and Equipment:

Semichon's carmine, stock solution (64)
Alcohol, ethyl, 70, 80 and 95 percent
Staining dishes
Alcohol, ethyl, acidified (add 1 ml conc. HCl to 100 ml of 70 percent alcohol)
Carbol-xylol, beechwood creosote, or aniline oil
Slides
Coverslips
Xylo-damar or balsam

Technique:

1. Mix Semichon's carmine stock solution in equal parts with 70 percent alcohol. Transfer specimens directly from 70 percent alcohol preservative to staining solution and allow to stain overnight.
2. Wash in several changes of 70 percent alcohol, 5 minutes each.
3. Place specimens in 70 percent acidified alcohol and destain until the specimens are a delicate pink color. For average-size specimens, destaining is usually accomplished in about 30 minutes. Change the alcohol if it becomes deep red in color.
4. Wash in several changes of 70 percent alcohol, 30 minutes each, until alcohol no longer acquires a pinkish color.
5. Dehydrate in successive changes of 80 and 95 percent alcohol, 30 minutes each.
6. Clear in aniline oil, carbol-xylol or beechwood creosote for 1 hour.
7. Mount in xylo-damar or balsam following method previously described for trematodes.

Mounting of Larval and Adult Nematodes

The cuticula of nematodes is quite impervious and the usual methods of fixation and dehydration are not satisfactory for preparation of permanent mounts of these worms. Simple and satisfactory techniques for mounts of this group include mounting in glycerin jelly, glycerin and Langeron's lacto-phenol. In these media, they may be mounted either stained or unstained.

Mounting in Glycerin:

Materials and Equipment:

Beakers
Staining dishes
Glycerin-alcohol (32)
Alcoholic eosin (3)
Slides
Coverslips, round. 18–22 mm
Camel's-hair brushes
Gold size
Asphaltum

Technique:

1. Place the specimens in a large beaker and pour a considerable volume of hot glycerin-alcohol over them. Nematodes are frequently mounted unstained, but if it is desired to stain them, a small amount of alcoholic eosin (enough to impart a light pink color) may be added to the glycerin-alcohol after the solution has cooled to room temperature.

2. Place the container in a 37° C. incubator and allow to stand until most of the alcohol has evaporated leaving the specimens in almost pure glycerin.
3. Prepare scrupulously clean slides. Center a slide either on a commercial type turntable or on a block in the head of a small centrifuge. Set the turntable or centrifuge in motion, dip a pointed camel's-hair brush into gold size and lightly touch the slide making a ring the outer dimensions of which about equal the outer dimensions of the coverslip to be used. The ring should be about $\frac{3}{16}$ of an inch in width. The brush should be lifted before the table or centrifuge stops turning. If the slide is turned too rapidly, the ring will spread and become uneven. If the brush is appressed too heavily against the slide, the ring will be very thick and the cell within will be too small. Ring the slides daily in the same manner until a cell of the desired depth has been made. Two or three applications are usually adequate. Slides should dry 24 hours after the last application of gold size. Just prior to making the mount, apply a fresh ring of gold size to serve as an adhesive for the coverslip.
4. With a camel's-hair brush, transfer a drop of the glycerin containing the specimens to the center of the cell on the slide. Spread and, if deemed necessary, add more glycerin. A slight excess of glycerin which will ooze out from beneath the margin of the coverslip is desirable in order to prevent entrapment of bubbles. Place the coverslip on the cell carefully lowering it gradually to a position where the margin of the ring is in contact with the outer margin of the coverslip. Seal the mount by carefully pressing at several points on the outer margin of the coverslip bringing it into firm contact with the freshly applied gold size.
5. Let slides stand 24 hours to allow time for the gold size to harden. At the end of 24 hours, any slides which show evidence of "leaks" should be set aside and the specimens remounted.
6. The margins of coverslips on slides which are well sealed should be gently wiped with lens paper to remove any excess glycerin, and the slides should be set aside to dry for an additional 24 hours.
7. Again place the slides on a turning device and with a camel's-hair brush apply a ring of asphaltum overlapping the slide and coverslip in such a manner that the preparation is sealed.

Permanent Mounts of Helminth Ova

Ringed Slide Technique:

Materials and Equipment:

Wire screens, #10, #30, and #60 mesh
Formalin, 5 percent
Slides, 1 x 3 inches
Coverslips, round, 18–22 mm
Camel's-hair brushes
Asphaltum-gold size mixture (2 parts asphaltum, 1 part gold size)
Shellac-gold size mixture (equal parts)
Medicine dropper
Applicator sticks

Technique:

1. Mix the fecal specimen thoroughly with a large volume of water and pass successively through #10, #30, and #60 mesh wire screens to remove large particles.
2. Concentrate ova by sedimentation technique described elsewhere and suspend the sediment in 5 percent formalin.
3. Place a clean slide on a turntable or secure it to the head of a small centrifuge and with a camel's-hair

brush, spin a ring of the asphaltum-gold size mixture. The outside diameter of the ring should be equal to the diameter of the round coverslip to be used. The ring should be about 2 mm in width. The asphaltum-gold size mixture should be about the consistency of glycerin. If it is too thick, thin it with xylol.

4. Set the slide or series of slides aside to dry for 24 hours. If a deeper cell is desired, one or more additional layers may be applied to the ring allowing a 24-hour drying period between each application. Large numbers of slides can be prepared and set aside indefinitely for future use.
5. At the time the mount is to be completed, an additional fresh ring of shellac-gold size mixture is spun on top of the asphaltum-gold size mixture. The shellac-gold size mixture should be about the consistency of glycerin. If too thick, thin with 95 percent alcohol.
6. With a medicine dropper, immediately apply a drop of the washed formalin fixed fecal specimen to the center of the cell. A slight excess which will be forced out when the coverslip is appressed to the preparation is desirable.
7. Touch a round coverslip to one side of the cell and lower it slowly onto the cell. The liquid should spread evenly and cover the entire cell without entrapping any bubbles.
8. After the coverslip is completely lowered, press the edges down gently with an applicator stick to bring the margin into firm contact with the soft shellac.
9. Set the slides aside to dry in flat position at room temperature.
10. The following day, any slides that show signs of evaporation due to incomplete sealing should be discarded. Remaining slides should again be placed on a spinning device and an additional ring of shellac and gold size spun overlapping the margin of the coverslip to seal it to the slide.
11. Following 24 hours or more of further drying, spin a ring of asphaltum-gold size mixture as a final coat.

Double Coverslip Technique:

Materials and Equipment:

Wire screens, #10, #30, and #60 mesh
Formalin, 5 percent
Medicine dropper
Coverslips, square, 22 mm
Coverslips, round, 18 mm
Slides, 1 x 3 inches
Permount or clarite
Applicator sticks

Technique:

1. Mix the fecal specimen thoroughly with a large volume of water and pass it successively through #10, #30, and #60 mesh wire screens to remove larger particles.
2. Concentrate ova by sedimentation technique described elsewhere and suspend the sediment in 5 percent formalin.
3. Place a drop of the formalized specimen on the center of a 22 mm square coverslip. Cover the specimen with an 18 mm round coverslip.
4. Place 2 or 3 drops of mounting medium on the center of a 1 x 3 inch slide. The exact amount of medium to use is determined by experience.
5. Invert the coverslips so the round coverslip is down and carefully place the preparation on the mounting medium.
6. Press gently around the margin of the square coverslip with an applicator stick to seal the preparation.
7. Dry in flat position until medium has hardened.

Glycerin Jelly Mounts:

Materials and Equipment:

Wire screens, #10, #30, and #60 mesh
Glycerin jelly (33)
Formalin, 5 percent
Centrifuge tube
Applicator sticks
Medicine dropper
Slides
Coverslips, round, 18-22 mm
Scalpel or razor blade
Camel's-hair brush
Asphaltum-gold size mixture (2 parts asphaltum, 1 part gold size)

Technique:

1. Comminute the specimen in a large volume of water and strain successively through #10, #30, and #60 wire screens.
2. Concentrate ova in the fecal specimen by sedimentation and suspend the concentrate in equal parts of warm glycerin jelly and 5 percent formalin in a centrifuge tube.
3. With an applicator stick, mix the suspension thoroughly and set it aside in a 37° C. incubator to evaporate the formalin. As the formalin evaporates, replace the fluid lost with warm glycerin jelly. This should be done about twice weekly over a period of several weeks.
4. When the ova are suspended in pure glycerin jelly, heat the suspension to about 50° C. and with a medicine dropper, transfer about 2 drops of the suspension to the center of a clean glass slide. While the medium is still warm, apply a coverslip and appress it slowly to the preparation.
5. Set the slides aside at room temperature until the glycerin jelly congeals. With a scalpel or razor blade, cut away any solidified gelatin which may have oozed out from beneath the coverslip.
6. With a camel's-hair brush, apply a coat of asphaltum-gold size mixture over the margin of the coverslip by spinning the slide on a turning device.

Chapter 3

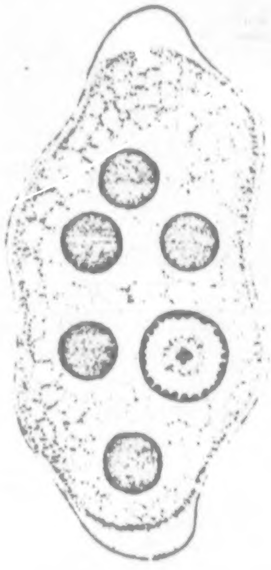
RECOGNITION OF INTESTINAL PARASITES

INTESTINAL AMOEBAE OF MAN

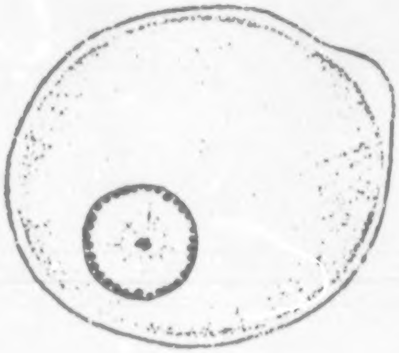
Finding and accurately identifying cysts and trophozoites of amoebae in the routine examination of fecal specimens is one of the most difficult tasks confronting the laboratory technician. The intestinal protozoa as a group derive their importance chiefly from the necessity for the differential diagnosis of those which may be of medical importance from those which are not. Since it is often impossible to rely on the clinical picture alone, the medical officer must rely upon laboratory findings in the diagnosis of many parasitic infections.

There are five species of amoebae which inhabit the human intestinal tract. Various stages as seen in both living condition and in iron-hematoxylin stained preparations will be described in the section which follows. Illustrations are based on iron-hematoxylin stained mounts.

Endamoeba histolytica

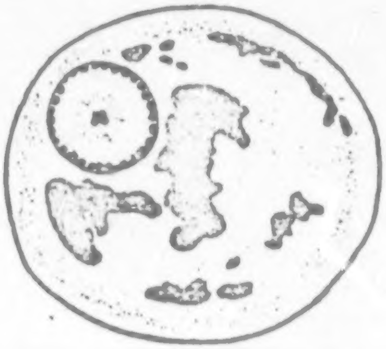

Trophozoite

Trophozoites. Trophozoites of this species are relatively large, about three or four times the size of a red blood cell. There are two distinct races, a small race and a large race. Smaller strains are not often associated with amoebic dysentery. In living condition about one-third of the amoeba consists of clear, hyaline ectoplasm and the remainder of the organism is finely granular endoplasm in which food vacuoles and the nucleus float about. When warm these amoebae are very active. They tend to travel in a straight line moving rapidly and advancing at either end a single, clear pseudopod, while ingested cells and the nucleus flow and roll about within them as though in a mobile liquid. Food inclusions consist of red blood cells or fragments of other body cells, principally epithelial cells and white blood cells from the intestinal tract. In this species ingested food never includes bacteria, starch, yeast, and the like. The nucleus in fresh preparations may be very indistinctly visible as a faint ring or entirely invisible. In stained preparations the ectoplasm and endoplasm are not differentiated into clearly defined zones. In all stages of development the nucleus is very nearly perfectly circular. The limiting membrane of the nucleus known as the nuclear membrane has on its inner lining a thin layer of rather evenly distributed chromatin granules, beadlike in arrangement. At the center of the nucleus is a small dot-like body known as the endosome or karyosome body. In the course of its transition from the active trophozoite to the dormant protected cystic stage several clearly defined stages can be identified.



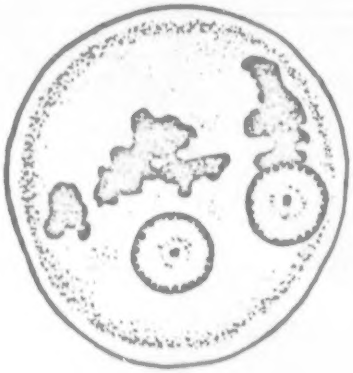
Pre-cyst

Pre-cysts. Progressing from the trophozoite stage the next stage observed is the pre-cyst. This is followed successively by the uninucleate, binucleate, and quadrinucleate cyst or mature form. As the organism commences encystment food inclusions are lost, the ectoplasmic zone becomes much more narrow and only occasional small pseudopods are observed. This is the typical pre-cyst.



Uninucleate Cyst

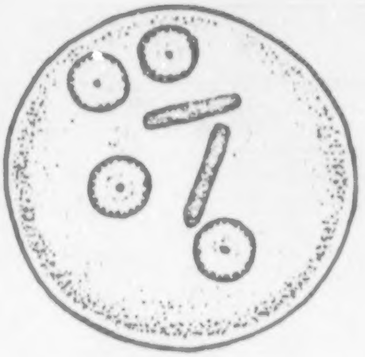
Uninucleate cysts. As the encystment process advances pseudopods are lost and a well defined cyst wall or protective covering is secreted. At this stage the organism is usually perfectly round or nearly so. Food inclusions are extruded and chromatoid bodies appear, several in number and of varying shapes and sizes. The one-nucleated cyst usually displays a rather broad, oval clear area which is known as the glycogen vacuole.



Binucleate Cyst

Binucleate cysts. As the organism progresses to the two-nucleate stage the glycogen vacuole becomes smaller or disappears and the chromatoid material begins to congregate into fewer and more uniform-sized masses.

Mature cysts. Further division of the nuclei occur resulting in the formation of the mature four-nucleate cyst. Throughout this gradual transition the nuclear characteristics remain unchanged. The dot-like karyosome body and the peripherally arranged bead-like chromatin granules are identical in all stages of development. Glycogen vacuoles are never observed in the four-nucleate cyst. Two principal diagnostic characters identify the mature cyst, namely, four nuclei typical of the species, together with one or two rod-like chromatoid bodies with parallel margins and smoothly rounded ends. In older cysts the chromatoid bodies may be completely lacking. Iron-hematoxylin stained chromatoid material and chromatin granules stain almost jet black.

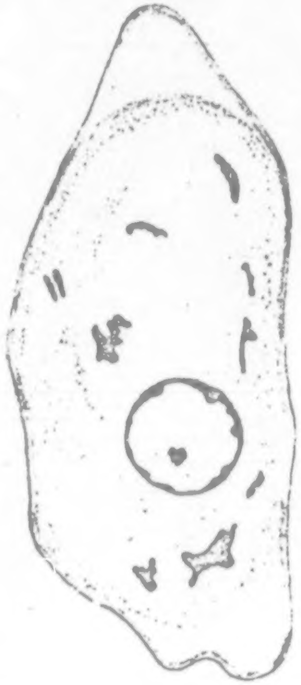


Mature Cyst

Endamoeba coli

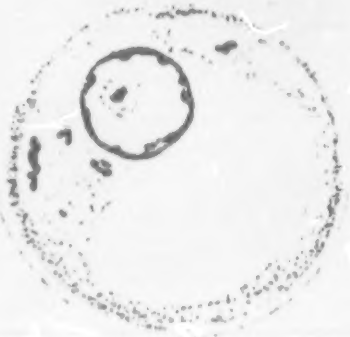
Trophozoites. There are several characteristics which differentiate this species from *E. histolytica* in the living trophozoites. This species is the largest of the intestinal amoebae and displays numerous types of food inclusions including bacteria, starch, portions of discharged body cells and yeast. Rarely red blood cells may occur within the cytoplasm, and then only in situations where other conditions or parasitic infections exist which cause hemorrhaging in the intestinal tract. Movement is sluggish and non-directional. Pseudopods are usually very narrow and composed mostly of endoplasm. The pseudopods are much less hyaline and much shorter and more blunt than those of *E. histolytica.* In unstrained preparations the nucleus is usually visible, appearing as a large refractile ring within which may be seen an eccentric hyaline mass, the karyosome body.

In stained preparations the nucleus of *E. coli* is very characteristic. On the inner periphery of the nuclear membrane the chromatin material is arranged in irregular masses. The karyosome body is large and almost always eccentric or off-center within the nuclear mass. As the organism progresses through various stages in the process of encystment several clearly recognizable transitional forms will be observed. These include



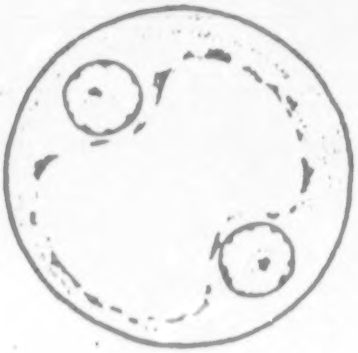
Trophozoite

precysts, uninucleate cysts, binucleate cysts, and the mature or eight-nucleated forms. The organism passes through a four-nucleate stage in the process of encystment. This stage is rarely seen, may be easily confused with the mature cyst of *E. histolytica*, and is, therefore, not discussed or illustrated here.



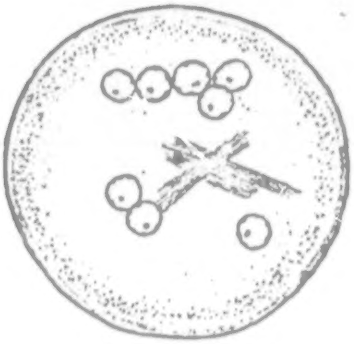
Pre-cyst

Pre-cysts. As the organism progresses from the trophozoite to the pre-cystic stage, food inclusions are extruded and numerous small masses of chromatin material appear. Very characteristically about one-third or more of the organism, may at this stage be occupied by a large clearly defined, rather transparent zone, the glycogen vacuole. The vacuole tends to push the nucleus off to one side of the pre-cyst. As encystment progresses a thick elastic cyst wall is gradually secreted about the organism.



Binucleate Cyst

Binucleate cysts. The next stage observed is that in which the single nucleus has divided resulting in the two-nucleate or binucleate cyst. Frequently in this stage the glycogen vacuole is considerably enlarged and occupies a large proportion of the cyst. The two nuclei are often observed in direct contact with the cyst wall and the large glycogen vacuole forms a characteristic "figure-of-eight" between the two nuclei. This stage of development is very characteristic for *E. coli.* In this stage the area between the glycogen vacuole and the cyst wall is usually filled with numerous irregular masses of chromatoid material.

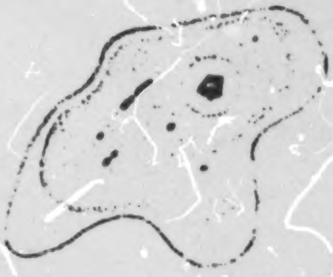


Mature Cyst

Mature cysts. Two further nuclear divisions occur resulting in the eight-nucleated mature form. At this stage most of the chromatoid material has disappeared and the remaining remnants consist of usually not more than two irregularly shaped masses the ends of which are frayed or "splinter-like" and the margins of which are not parallel to each other. Throughout all stages of development the nuclear elements diagnostic of the species remain the same, namely, a large eccentrically placed karyosome body surrounded by a halo of fine granules, and a clearly defined nuclear membrane the inner periphery of which is lined with irregular masses of chromatin material.

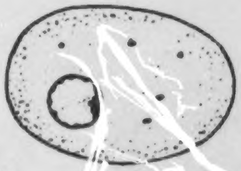
Endolimax nana

Trophozoites. This species is very small, rarely averaging a diameter greater than that of a red blood cell, and occasionally even smaller. Movement is very sluggish and the pseudopods are narrow. Generally, movement is non-directional and pseudopods are extruded in various directions with minimal directional motion. The cytoplasm is usually filled with numerous vacuoles containing bacteria and the cytoplasm is heavily granulated. If the nucleus is visible it can be seen in hazy outline as a rounded hyaline mass. In stained preparations nuclear characteristics, size, and character and abundance of food inclusions, are the principal diagnostic characters by means of which the species can be recognized. The nuclear membrane is often invisible and the nucleus is characterized by a karyosome body which displays an outline much like a "chunk of coal." It is often trapezoidal in shape and may show occasional lobulation. The area between the ill defined nuclear membrane and the karyosome body is a "halo-like" narrow clear zone free of granulation.



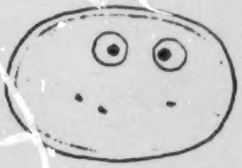
Trophozoite

Uninucleate cysts. As the organism goes into the cystic stage food inclusions are lost and the cytoplasm becomes more granular. Gradually as the transitional stages occur a thin elastic cyst wall is secreted. In the uninucleate stage and subsequent changes as the cysts mature the karyosome body becomes smaller and more dense and the nuclear membrane can be discerned.

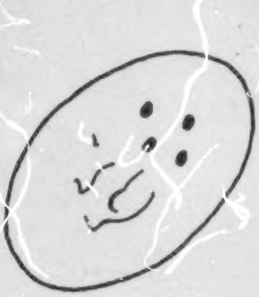


Uninucleate Cyst

Binucleate cysts. In this stage nuclear characteristics are similar to those observed in the one-nucleated form. The nuclei generally lie close together at one pole of the cyst and karyosome bodies are frequently eccentric and in close contact with the nuclear membrane.



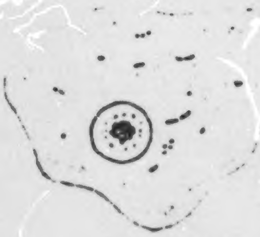
Binucleate Cyst



Mature Cyst

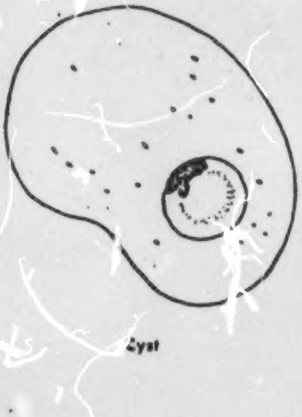
Mature cysts. The mature four-nucleate stage is the form most frequently seen in routine fecal examinations. In stained preparations the nuclei are generally clustered close together, four in number, give much the appearance of four "eyes" in quadrate arrangement. The karyosome bodies and their surrounding nuclear membranes are oval in shape, the former filling about one-half the total area of the nucleus. Cysts are sometimes round but most often ovoid in shape. Chromatoid bodies are not seen in this species. The endoplasm often contains a number of small refractile granules of a substance known as volutin. Young cysts may also contain small masses of glycogen.

Iodamoeba butschlii



Trophozoite

Trophozoites. In the living trophozoites which are about one and one-half times the diameter of a red blood cell, movement is sluggish, pseudopods are leaf-like, greenish-yellow in appearance and broadly clear margined. The trophozoites are easily confused with those of small strains of *E. histolytica.* They are most readily differentiated from the latter by the nature of food inclusions which in the present species consists principally of bacteria.

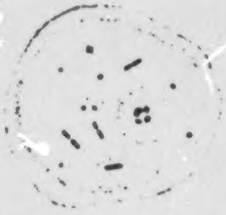


Cyst

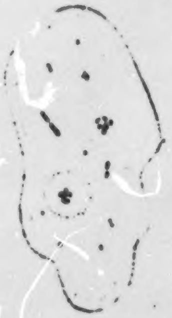
Cysts. This is the only species of intestinal amoebae of man in which a single nucleus persists throughout both the trophozoite and cystic stages. A clearly defined pre-cystic stage is not recognized. In the cystic stage the nucleus differs considerably from that of the trophozoite. In both stages the nuclear membrane is distinct. In trophozoites the irregular karyosome body is centrally situated and surrounded by a halo whereas in cysts the karyosome lies at or near the nuclear membrane. The nuclear membrane of cysts in well stained preparations displays irregularly spaced chromatin granules which are margined by a "scallop-like" fringe surrounding the membrane. The cyst is also characterized by a large glycogen vacuole which stains dark mahogany with iodine stain and appears as a clearly defined zone surrounded by a halo in hematoxylin stained preparations. Within the cytoplasm of the cyst there will frequently be observed small dark masses which to the inexperienced observer may be mistaken for bacteria but which are actually inclusions known as volutin granules. These are similar in consistency to chromatoid bodies seen in other species, but much smaller. Another important characteristic of the cyst is that it is generally neither round nor oval in shape but rather irregular in outline presenting no characteristic form.

Dientamoeba fragilis

Trophozoites. Living trophozoites of this species in fresh preparations extrude clear pseudopods and the movement of the organism is active and progressive. With a drop in temperature the organism soon becomes inactive, rounds up and quickly degenerates. In contact with tap water they quickly rupture and disintegrate. The cytoplasm is frothy granular in appearance and the ectoplasmic and endoplasmic zones are sharply demarked. Food inclusions generally consist of bacteria. About eighty percent of the organisms of this species observed in routine fecal examinations bear two nuclei. The nucleus consists of an aggregation of separate granules four to eight in number, generally not over five. The nuclear membrane is usually indistinct or often invisible and there is no chromatin lining on the inner periphery of the nuclear membrane. This is the only species of intestinal amoebae of man in which no cystic stage is known, thus far only trophozoites have been observed.



Trophozoite
(Uninucleate)



Trophozoite
(Binucleate)

KEY TO THE TROPHOZOITES OF THE AMOEBAE OF MAN

1. Occur in the intestine 2
   Occur in the mouth .... *Endamoeba gingivalis*
2. Typical trophozoite with a single nucleus 3
   Typical trophozoite with two nuclei .... *Dientamoeba fragilis*
3. Nucleus with comparatively large karyosome and little if any peripheral chromatin 4
   Nucleus with comparatively small karyosome and distinct peripheral chromatin 5
4. Karyosome spherical, usually central in trophozoite nucleus, nuclear membrane delicate and often indistinct .... *Iodamoeba butschlii*
   Karyosome often irregular, block-like, and eccentrically placed in nucleus. Nuclear membrane distinct .... *Endolimax nana*
5. Nucleus with very small, central karyosome and fine peripheral chromatin granules .... *Endamoeba histolytica*
   Nucleus with larger, typically eccentric karyosome and coarse, irregularly distributed peripheral chromatin granules .... *Endamoeba coli*

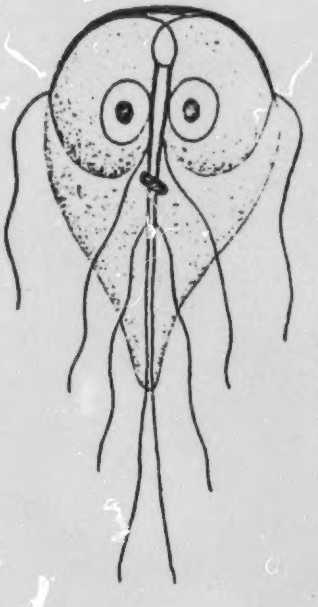
KEY TO THE CYSTS OF THE AMOEBAE OF MAN

1. Mature cysts with more than one nucleus 2
   Mature cysts with a single nucleus .... *Iodamoeba butschlii*
2. Mature cysts containing four nuclei 3
   Mature cysts containing eight nuclei .... *Endamoeba coli*
3. Nuclei with a very small, dot-like centrally placed karyosome and fine bead-like peripheral chromatin. Chromatoids generally present. Cysts ovoid .. *Endamoeba histolytica*
   Nuclei with large, irregular, block-like karyosome. Peripheral chromatin generally lacking. Chromatoids absent. Cysts elliptical .... *Endolimax nana*

THE INTESTINAL AND VAGINAL FLAGELLATES AND CILIATES OF MAN

The common diagnostic character found in this group of protozoan parasites is that all possess locomotor organs known as flagella. They differ from the amoebae in that their body contours are fairly constant. A number of definite structures known as organelles which have specialized functions occur in the flagellates. These include axostyles which are rigid supporting rod-like organelles, the flagella and undulating membrane for locomotion, and a cytostome or protozoan mouth and gullet, and *blephoraplasts* which function as energy control centers. All organelles are not found within each species within this group.

Giardia lamblia



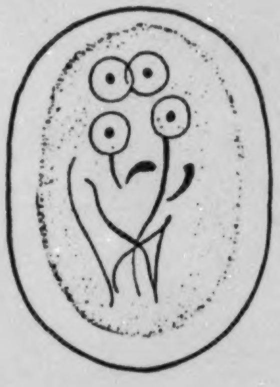
Trophozoite

Trophozoites. In fresh preparations the trophozoite of this species is actively motile and combines various movements including rotation, rocking, and forward and backward motion in rapid succession. In shape this flagellate resembles a longitudinally cut pear. Its length is more than double that of a red blood cell and its width is slightly greater than the diameter of a red cell. It is shaped from dorsal to ventral surface much like a saucer, being dorsally convex and ventrally concave. The concave side forms an ovoid sucking disc by means of which it is capable of adhering to surfaces.

The detailed morphology of this species is visible only in hematoxylin stained preparations. This is the only bilaterally symmetrical protozoan infecting man. That is to say, right and left halves are identical to each other in the trophozoite and all structures except the central longitudinal supporting structure, the axostyle, are duplicated. Locomotor organs consist of eight flagella, a pair of which arise from anteriorally situated granules known as blepharoplasts. Additionally two pair of flagella originate from the blephoraplasts and are directed backward. A fourth pair originates at the end of the longitudinal axostyle, the latter forming the framework of skeleton of the organism. In the anterior third of the body there are two nuclei, one on either side of the axostyle. These exhibit clearly defined nuclear membranes and bear prominent ovoid karyosome bodies. Both the karyosome and the nucleus are ovoid in shape. Transversely a little below the center of the organism will generally be seen two rod-like structures lying more or less diagonally across the center of the body. The cytoplasm is finely granular and never contains food inclusions, since this organism obtains its nourishment by absorption of digested food through the body wall.

Cysts. In temporary mounts the cysts are readily recognized, particularly if iodine or MIF stains have been added to the preparation. The unstained cysts appear as broadly ovoid symmetrical bodies in which are easily observed four ovoid colorless or hyaline structures with a refractile center, these representing the karyosome body and nucleus respectively. This organism is characterized both in stained and unstained preparations, employing either permanent or temporary stains, by the fact that there is a marked shrinking away of the organism from the thick double walled cysts. The contained organism occupies only about two-thirds of the total area within the cyst.

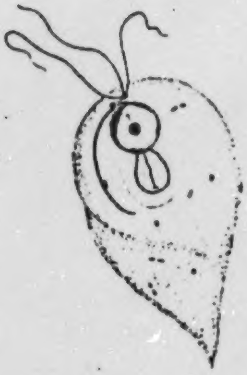
In hematoxylin stained preparations the nuclei stand out very clearly as four "eye-like" ovoid objects similar in morphology to the nucleus seen in the trophozoite stage. These nuclei are generally grouped, four in a cluster, fairly close to each other in the anterior end of the organism. The remains of the axostyle also appear as a curved or S-shaped rod running down the center of the cyst. Oftentimes delicate fibrils representing the remmants of the flagella observed in the troprozoite stage are also seen in the cyst. When a patient is passing these cysts they are usually found in tremendous numbers although not regularly—they are frequently eliminated from the body in "showers." It is not uncommon to observe several dozen cysts in a low-power field of mag-



Cyst

nification. Reproduction in this species very likely occurs first through the process of encystment followed by excystment within the host harboring the trophozoite. Each time this multiplicative process occurs the number of parasites is quadrupled. This is believed to account for the large numbers of parasites often encountered. Thus encystment serves as both a multiplicative process as well as a process by means of which the organism is protected during periods of adverse environmental conditions.

Chilomastix mesnili

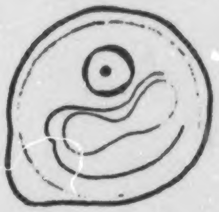


Trophozoite

Trophozoites. In fresh preparations the trophozoites of this species are very actively motile progressing in a jerky spiral fashion with a directional line of movement. The cytoplasm is faintly greyish-green in color. Movement is accomplished by means of flagella which can be observed only in sluggishly motile trophozoites or in stained preparations.

In hematoxylin stained preparations various external and internal structures are clearly visible. The cytoplasm is finely granular and contains numerous food inclusions, principally bacteria and starch. Other prominent internal structures include the anteriorly placed nucleus and the primitive mouth or cytostome. The cytostome appears as a cleft in the body wall originating anteriorly and extending posteriorly almost one-half the length of the body. This organelle is margined by a short fibril on one side and a longer heavy fibril having the contour of a half "figure-of-eight" on the other. The nucleus is situated in the extreme anterior end and has a well defined nuclear membrane the inner periphery of which is thickened with heavy granules some of which coalesce on one side of the nucleus into a rather broad band. The karyosome body is small, dotlike, and slightly eccentric in position. Immediately above the nucleus in the anterior end of the organism there is a dot-like structure or blepharoplast from which the flagella arise. Three flagella are directed anteriorly and a fourth extends posteriorly about one-half the length of the body. There is no undulating membrane.

Cysts. Stained cysts of this species are ovoid in shape about equal in length to a red blood cell and about two-thirds as wide. The most characteristic morphological structure is a protuberance on one margin giving the cyst a "lemon"-shaped appearance. This protuberance together with a clear ungranulated area beneath and the characteristic shape of the cyst are the most important identifying characters in this stage. Cysts contain a single nucleus situated near the center and bearing a prominent karyosome body and a clearly defined nuclear membrane on which are distributed alternately thin and thickened areas of peripheral chromatin. Remnants of the trophozoite stage contained in the cyst usually include an "hour-glass"-shaped cystostome and remnants of flagella which appear as hair-like fibrils.

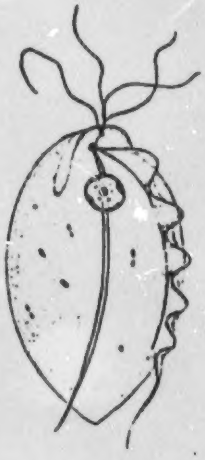


Cyst

Trichomonas hominis

Trophozoites. In fresh preparations, this species exhibits a wobbly progressive motion which is brought about by the active vibration of the flagella and the undulating membrane. In order to observe the trophozoite light must be cut down considerably since the organism is very hyaline, clear, and finely granular. The nucleus and other internal structures are not visible in the colorless finely granular cytoplasm.

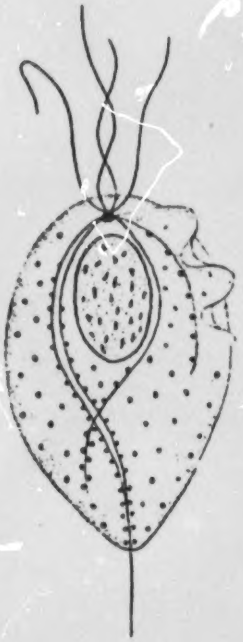
In hematoxylin stained preparations various internal and external structures are readily observed. The organism is pear-shaped and bears three to five anteriorly directed flagella originating from the blepharoplast, and a single posteriorly directed flagellum which forms the margin of the undulating membrane and terminates in a free flagellum in the posterior end of the body. The prominent undulating membrane is a thin, clear, transparent, fin-like organelle running throughout the entire length of the body. The outer margins of this fold are thrown into numerous ripples. The supporting structure of the organism is the axostyle which originates at the



Trophozoite

blepharoplast at the anterior end of the body and runs through its length terminating beyond the posterior margin in a fine point. A primitive mouth or cytostome is present in the anterior third of the body. The nucleus is prominent and the thin nuclear membrane encloses a clear area bearing several prominent granules. The dot-like karyosome body is eccentric and stands out conspicuously. No cystic stage is known in this species.

Trichomonas vaginalis



Trophozoite

Trophozoites. In fresh urethral or vaginal smears this species is readily visible under high dry magnification. The presence of motile flagellated organisms in discharges from the vagina in females and the urethra in males is diagnostic for this species since no other flagellates occur in the genito-urinary tract of man. This species may also be observed in routine urinalysis on both men and women. The infection is, however, much more common in females.

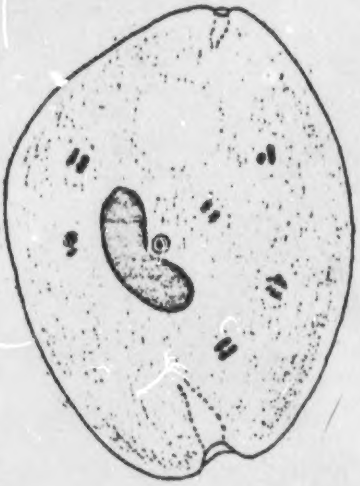
The cytoplasm is uniformly granular and food inclusions are lacking. Motion is zig-zag and directional. Under reduced light the organisms are highly refractile and their pear-shaped form and characteristic movement is readily observed.

In stained preparations various internal and external morphological characters can be readily identified. There are four anteriorly directed flagella arising from the blepharoplast and a fifth flagellum is directed posteriorly and forms the margin of the undulating membrane. This flagellum terminates as a free fibril about halfway down the lateral margin of the body wall. The nucleus is usually elongated, with a small karyosome centrally situated within the nucleus. The axostyle is relatively slender and curved as it passes alongside of the oval nucleus. The area between the karyosome body and the nuclear membrane is clear. In stained preparations there are numerous rod-like prominent dark staining granules evenly distributed throughout the cytoplasm. These are known as volutin granules and are believed to be the counterpart of chromatoid bodies in other protozoans. The supporting axostyle originates in the blepharoplast and terminates posteriorly in a rather long free portion protruding from the body wall. No cystic stage is known in this species.

Balantidium coli

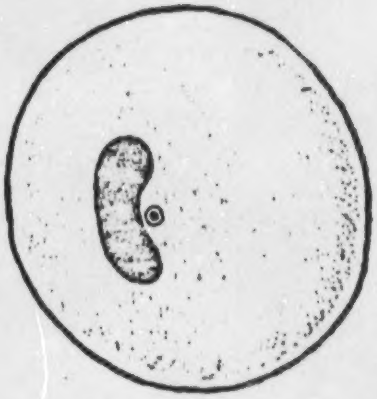
Trophozoites. This is the largest protozoan of man. It differs most markedly from the flagellates in the fact that locomotion is by means of cilia. The motile trophozoites in fresh preparations are large, ovoidal, slightly greenish bodies, displaying directional, rapid, smooth gliding movements. Off to one side of the anterior end is a deep cleft which is the cytostome or primitive mouth. The outer surface of the body is covered with a delicate pellicle on the surface of which are implanted in regular rows, numerous short cilia. Food vacuoles are very numerous and consist almost entirely of starch. In living condition one small anterior and a large posterior excretory vacuole can be seen, first enlarging and then expelling their contents at regular intervals.

In stained trophozoites the surface covering of longitudinal rows of cilia can be seen. The most prominent internal structures are the two nuclei, a large kidney-shaped macronucleus in the concave surface of which lies the small dot-like micronucleus. This species is a heavy feeder and the cytoplasm is filled with food inclusions.



Trophozoite

Cysts. Cysts are not commonly encountered. When seen they are large double-walled spherical bodies eight to ten times the diameter of a red blood cell. The cytoplasm is uniformly heavily granulated and lacks food inclusions. Macro- and micronucleus stain clearly and are identical in outline to those seen in the trophozoite stage.



Cyst

Table 1. A Comparative Table of the Medically Important Amoebae of Man

	Diagnostic Characters	*Endamoeba histolytica*	*Endamoeba coli*	*Dientamoeba fragilis*
TROPHOZOITES	Size	Quite variable, but generally between 20-30μ. There are at least two distinct races based on size. Broadest limits of size range from 15-60 microns.	Quite variable but within the limits of 15-60μ.	Small species averaging 9μ in diameter.
	Motility	One or two blunt pseudopodia are usually present in normal fresh preparations. Movement is rapid and directional.	Less actively motile than *E. histolytica*. Movement is not directional. Pseudopods form slowly and are short and blunt.	In fresh warm specimens pseudopods clear. Movement active, progressive. As temperature drops it becomes inactive and soon degenerates.
	Cytoplasm and inclusions (in fresh preparations).	Ectoplasm and endoplasm clearly differentiated with former broad and glass-clear. Food inclusions often consist of red cells.	Less hyaline, ectoplasm not clearly differentiated from endoplasm. Food inclusions limited mostly to bacteria, starch and cysts of small protozoans.	Cytoplasm frothy granular in appearance. Ectoplasm usually sharply demarked from endoplasm.
	Nuclei in stained preparation.	Nucleus is ring-like, off center in position and indistinct. On the lining of the nuclear membrane is a thin layer of chromatin, beadlike in arrangement. At center of nucleus is a small dot-like karyosome.	Nucleus is ring-like, near center of organism and can almost always be seen in fresh specimens. Karyosome is large and distinct. Chromatin on inner periphery of nucleus in heavy masses.	Nucleus has at its center a cluster of 4-8 deep-staining granules one of which stains more deeply than the others. Trophozoites generally two-nucleated.
	Location within host	Commonly the sub-mucosal tissue of the large intestine. Carried by the circulatory system it may infect the liver, lung, brain, and other organs. Occasionally the skin may be involved.	In the lumen of the intestine only. Organism is never invasive.	Occurs in the lumen of the intestine only and produces no lesions.
	Pathogenicity	From medical viewpoint this is the most important amoeba of man. With or without clinical manifestations infection with this species is known as amoebiasis. While most infections are asymptomatic a small percentage are clinical.	Non-pathogenic. Of importance only because it is easily confused with *E. histolytica*.	Strong evidence that this form may be pathogenic. Gastrointestinal disturbances including diarrhea are sometimes associated with infections with this species.

Table 1. A Comparative Table of the Medically Important Amoebae of Man—Continued

	Diagnostic Characters	*Endamoeba histolytica*	*Endamoeba coli*	*Dientamoeba fragilis*
CYSTS	Size and shape	Cysts are formed within the lumen of the intestine. They are roundish and 7–18µ in size and are generally smaller than those of *E. coli*.	Cysts are roundish, 15–20µ in diameter.	Cysts are not known in this species.
	Number of nuclei	Young cysts, 1 or 2. Mature cysts, 4.	Young cysts 1, 2, or 4. Mature cysts generally 8, sometimes 16.	
	Structure of nuclei and staining properties.	Nuclei as in trophozoites above. Cytoplasm stains weakly with iron-haematoxylin.	Nuclei as in trophozoite above. Cytoplasm stains deeply with iron-haematoxylin.	
	Chromatoid bodies, glycogen vacuoles and other inclusions.	Thick or slender bars with parallel sides and smoothly rounded ends. Chromatoids not always present. Glycogen vacuole is large in uninucleated immature cysts.	Glycogen vacuole large in immature binucleate cysts. Chromatoid bodies not often observed, splinter-like (like small needles), single or in clusters.	
		Iodamoeba butschlii	*Endolimax nana*	*Endamoeba gingivalis*
TROPHOZOITES	Size	Trophozoites average 9 to 11µ in diameter. Species is usually larger than *E. nana*.	Very small species averaging 7–9µ, often even smaller.	Extremely variable in size. Different authors' measurements range between 6–60µ. Average size is between 20 and 30µ.
	Motility	Movement is sluggish and pseudopods leaf-like in appearance and broadly clear-margined.	Movement is sluggish as in *E. coli*.	Pseudopods usually broad and rounded, resembling large blisters. Movement is generally rapid in various directions.
	Cytoplasm and inclusions (in stained preparations).	Finely granular and greyish with bacterial inclusions.	Usually contains numerous vacuoles filled with bacteria; appears highly granular.	The endoplasm contains leukocytes, bacteria, tissue cells, food vacuoles and according to some observers, occasionally red cells.
	Location within host.	In the lumen of the intestine only. Organism is never invasive.	In the lumen of the intestine only.	In the tartar at juncture of teeth with gums, as well as in pus pockets between the gums and the peridontum.

Table 1. A Comparative Table of the Medically Important Amoebae of Man—Continued

	Diagnostic Characters	*Iodamoeba butschlii*	*Endolimax nana*	*Endamoeba gingivalis*
TROPHOZOITES	Pathogenicity........	Nonpathogenic..........	Non-pathogenic but cysts are easily confused with those of *E. histolytica*.	This form may be pathogenic and is often found in mouths of persons suffering from pyorrhea. It should be regarded as a probable pathogen.
CYSTS	Size and shape......	Usually uninucleate, rarely binucleate.	Young cysts, with 1 or 2 nuclei; mature cysts 4 nucleated, very rarely 8 nucleated.	Cysts are not formed in this species.
	Structure of nuclei and staining properties.	Nuclei as in trophozoite above excepting that more than one mass of chromatin may be present. When this is the case the masses are frequently interconnected by strands.	Nuclei as in trophozoite.	
	Chromatoid bodies, glycogen vacuoles and other inclusions.	Chromatoid bodies lacking. Large glycogen vacuole present which stains dark brown with iodine.	No true chromatoid bodies. Glycogen masses occasionally visible in iodine stained preparations.	

Table 2. A Comparative Table of the Medically Important Intestinal and Vaginal Flagellates

	Diagnostic Characters	*Giardia lamblia*	*Chilomastix mesnili*	*Trichomonas vaginalis*	*Trichomonas hominis*
TROPHOZOITES	Size and shape..	About 8μ wide by 15μ in length. Pear-shaped in dorsal view.	About $10-15\mu$ in length and slightly narrower (almost spherical).	Usually $15-18\mu$ in length by $5-15\mu$ in breadth. Shape variable, usually pyriform.	About $8-15\mu$ long by $3-5\mu$ in breadth. Shape variable, usually pyriform.
	Number of flagella.	8	3 anteriorly directed and 1 short posteriorly directed.	4	3 or 4.
	Internal morphology.	Two nuclei, two axostyles and two blepharoplasts present. Organism is bilaterally symmetrical. A fused pair of deeply staining curved bars lie diagonally just below center of the organism. The nuclei contain centrally located large deeply staining karyosomes.	Cytoplasm grayish, containing numerous vacuoles and food particles. Round or rarely oval nucleus lies close to anterior end and bears well defined small slightly eccentric karyosome.	Coarse chromatic granules present. Nuclear chromatin in fine granules, without definite karyosome. Ingested bacteria present.	No chromatic granulation of cytoplasm. Nuclear membrane encloses clear space with conspicuous karyosome.
	Undulating membrane.	Absent.	Absent.	One-third to full length of body but not ending in a free trailing flagellum.	Full length of body and ending in a free trailing flagellum.
CYSTS	Size and shape..	Ellipsoidal in shape. Average length is $9-12\mu$ and average breadth is $6-8\mu$.	Lemon-shaped with knob at interior end, rarely oval in shape. Bluish-green in color, averaging about 8μ in length.	Cysts are unknown in this species.	Cysts are unknown in this species.
	Nuclei..........	Usually 4 in number, occasionally 8. Nuclei usually in anterior end of cyst. Numerous inclusions representing remnants of trophozoite structure present.	Single nuclesu present. Nuclear chromatin tending to condense into several peripheral masses. Fairly large eccentric karyosome present. Numerous inclusions representing remnants of trophozoite structures present.		

CESTODES OF MAN (Tapeworms)

Whole specimens or portions of cestodes may be submitted to the parasitology section of the laboratory for species identification. These may be passed by the patient spontaneously or they may be eliminated following the administration of anthelminthics. The technician should be able to identify the eggs, proglottids, and the heads of cestodes, all of which possess important diagnostic characters.

All species of cestodes discharge eggs which, when fertilized and normally developed, contain a motile six-hooked or hexacanth embryo. These eggs may be passed singly, in packets containing several eggs surrounded by a membrane, or within mature gravid proglottids which become detached from the posterior end of the strobila or chain of the main body of the worm. In fresh preparations discharged proglottids are often actively motile.

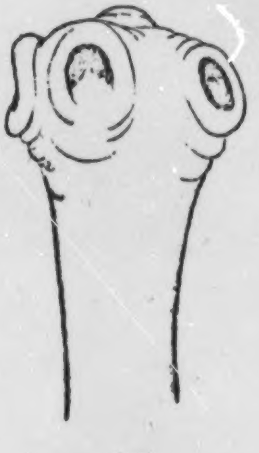
Morphological characters of attachment organs are very useful in species identification. In the majority of tapeworms organs of attachment consist of various combinations of hooks and suckers. In some species hooks are entirely lacking, while in others there are varying numbers of hooks generally arranged in rows on the anterior-most portion of the worm, the rostellum. The majority of human tapeworms possess four rounded cupped suckers arranged in a quadrangle at the widest portion of the head.

The principal identification character for species recognition of proglottids is the outline of the egg-filled uterus in gravid segments and the morphology of the male and female reproductive systems in immature segments. Other diagnostic characters include number and location of genital pores, shape of the lateral margins of segments, and the comparative distance between the anterior and posterior and right and left margins of the segment.

With the exception of one species the ova of tapeworms can be identified by variations found within the shell or embryophore. Shells vary in thickness, coloration, and the presence or absence of polar fibrils and striations. With the exception of one species all contain a fully developed six-hooked embryo.

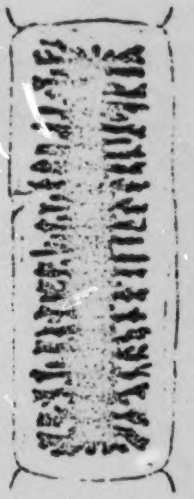
Diagnostic characters for species identification of tapeworms are found in the head, proglottids and eggs. Diagnostic characteristics are described and illustrated in the section which follows.

Taenia saginata



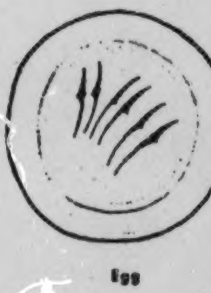
Head

Adult. The head of this tapeworm is characterized by the fact that the rostellum is completely lacking and that it bears no attachment hooks. The attachment organs consist of four cup-shaped hemispherical suckers one each at the four corners of the head. Mature proglottids are slightly broader than long while the gravid ones are considerably more narrow and about three times as long as the broadest portion which is at the center of the segment. In gravid proglottids the central continuous tube of the uterus and its lateral branches, fifteen to twenty in number, are filled with eggs.



Proglottid

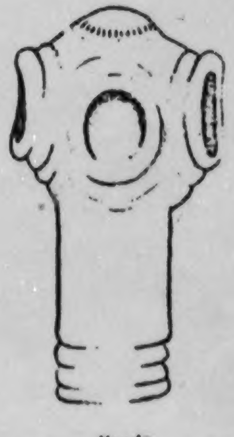
Eggs. The eggs of this species cannot be distinguished from those of the closely related human tapeworm, *Taenia solium.* They are referred to simply as "Taenoid" eggs without reference to species. Taenoid eggs are characterized by the presence of longitudinal striations in the shell or embryophore. The shell is thick and there is a clear line of demarkation between the contained hexacanth embryo since the embryo does not completely fill the egg. The striations of the egg are very fine and close together and readily observed under reduced light.



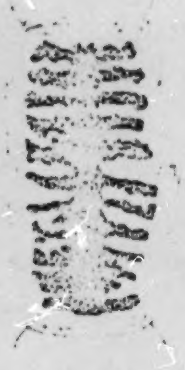
Egg

Taenia solium

Adult. The head bears a prominent rounded rostellum which is armed with a single row of hooks which taper to a fine point and are armed with a lateral denticle at about the middle. Hooks are twenty to thirty-two in number and are alternately large and small in a regular pattern on the margin of the rostellum. The scolex is quadrate in shape and bears at each corner deeply cupped prominent suckers. Mature proglottids are slightly wider than long and contain male and female reproductive systems typical of this genus. The egg-filled and considerably elongated gravid proglottids in the terminal portion of the chain display the characteristic uterine



Head

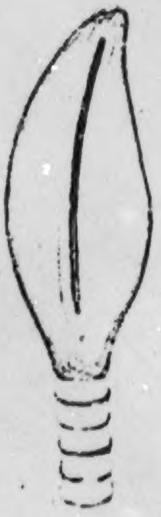


Proglottid

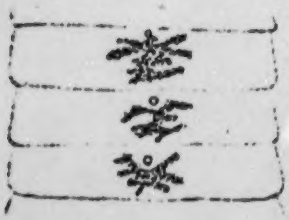
branching diagnostic for this species. The uterus consists of a single longitudinal centrally situated tube from which arise laterally on each side seven to thirteen lateral branches (generally about nine).

Eggs. Taenoid in type. See *T. saginata* above.

Diphyllobothrium latum



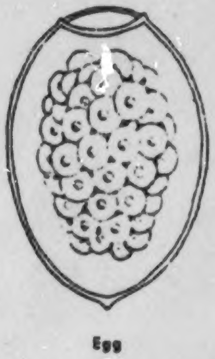
Head



Proglottid

Adult. The scolex is small and spoonlike in shape, bearing two longitudinal slits or "bothria" which serve as organs or attachment. Neither hooks nor true suckers are present in this species. Proglottids are very short in the anterior-posterior dimension and about four times as wide as long. In both mature and gravid proglottids the principal diagnostic characters are the centrally situated "rosette" shaped uterus and the genital pore which is in the center of each segment.

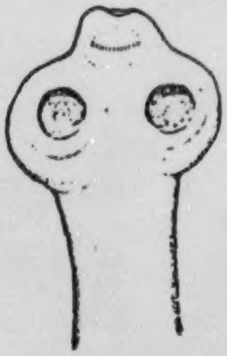
Eggs. The eggs of this species are broadly ovoidal in shape displaying a contour very similar to that of a hen's egg. The shell is moderately thick, and golden-brown in color when discharged by the patient due to absorption of pigments in the intestinal tract. They contain a partially developed embryo at the time of discharge, generally in about the thirty-two cell stage of development. At its anterior pole the egg bears a prominent cap or operculum and the posterior pole is characterized by a small knob-like protuberance which, however, is not always present.



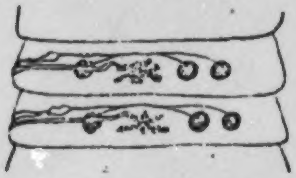
Egg

Hymenolepis nana

Adults. The head is diamond-shaped and bears hemispherical suckers at the four corners. The rostellum is short and armed with twenty to thirty spine-like hooks of equal size arranged in a single ring. The rostellar neck is short and capable of invaginating the rostellum into the head. Either invaginated or evaginated scolices may be seen in routine examinations. In mature stained proglottids the most prominent diagnostic feature which will be observed is the presence of three ovoid irregularly spaced testes along the lower margin. The proglottids are short in anterior-posterior dimension and three or four times as wide. Gravid proglottids are completely filled with eggs and the outlines of the uterine branches are obliterated.

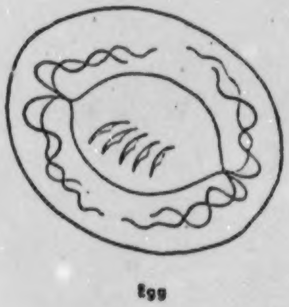


Head



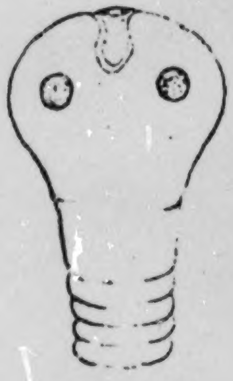
Proglottid

Eggs. The embryophore is very thick and bears prominent knobs at both poles from which arise four to eight filaments which radiate out as fibrils within the egg shell. The area immediately beneath the cortex is highly granular and proceeding inwardly granulation gradually decreases toward the inner periphery of the shell.



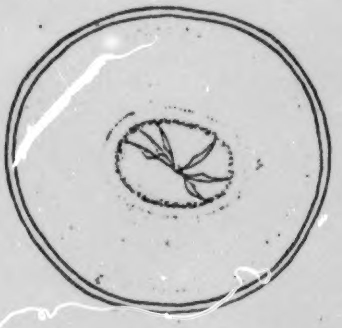
Egg

Hymenolepis diminuta



Head

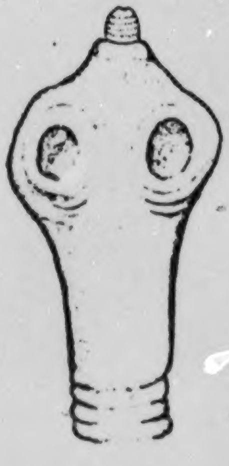
Adult. The scolex is small and provided with four comparatively small deeply cupped suckers. The rostellum is triangular in shape, unarmed and often invaginated into the scolex. Proglottids are very similar to those of *H. nana* from which they cannot easily be differentiated. In the gravid proglottids the principal diagnostic character is the presence of three irregularly spaced ovoid testes situated along the lower margin. The gravid proglottid is completely filled with eggs obliterating the outlines of the uterus. Proglottids are very short, and about three or four times as wide.



Egg

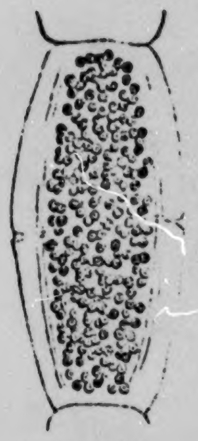
Eggs. Eggs are slightly ovoid in shape and covered with a very thick double shell. The inner and outer margins of the protective covering of the embryophore (inner shell) display numerous irregular lobulations. Polar knobs are present but polar filaments lacking.

Dipylidium caninum



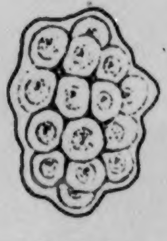
Head

Adult. The head is quadrate in shape and bears four deeply cupped suckers and a prominent club-shaped retractile rostellum. The rostellum is armed with one to seven circles of fine spine-like hooks, the number of rows varying with the age of the worm. The anterior spines are the longest and posterior the shortest. Mature proglottids are about as broad as long. As the proglottids mature and become filled with packets of eggs the margins become distended and the segments elongate assuming the shape of a "pumpkin seed." This species is bilaterally symmetrical bearing double sets of reproductive organs within each proglottid and genital pores are situated at about the center and on both sides of the segment.



Proglottid

Eggs. Eggs are generally passed in mother capsules or packets containing varying numbers of eggs up to about twenty. The single shell is relatively thick and sparsely and uniformly granulated throughout. Eggs are usually discharged in packets but occasionally may be observed singly. The most frequent method of passage of eggs from the host occurs through shedding of gravid proglottids from the posterior end of the strobila or chain.



Egg Packet

Table 3. A Comparative Table of the Cestodes of Medical Importance

Diagnostic Characters	*Taenia saginata*	*Taenia solium*	*Diphyllobothrium latum*
Common name . . .	Beef tapeworm of man	Pork tapeworm of man	Fish tapeworm of man.
Attachment hooks	Unarmed rostellum	Rostellum with double row of attachment hooks.	Lacking.
Attachment organs.	4 suckers	4 suckers	2 longitudinal slits or bothria.
Length	12–36 feet	12–14 feet	As long as 50 feet.
Number of proglottids.	As many as 2,000	1,000 or less	300–4,000.
Recognition characters of sexually mature proglottids.	Gravid proglottid elongated with uterus consisting of 15–30 dichotomous (tree-like) branches. Genital pores irregularly alternating on margin of each proglottid.	Gravid proglottid elongated with uterus consisting of 12 or fewer dendritic branches. Genital pores irregularly alternating on margin of each proglottid.	Gravid proglottid narrow in anterior-posterior dimensions and containing a "rosette-like" uterus at center of segment. Genital pore present on each proglottid on mid-ventral line.

Table 3. A Comparative Table of the Cestodes of Medical Importance—Continued

Diagnostic Characters	*Taenia saginata*	*Taenia solium*	*Diphyllobothrium latum*
Ova	Approximately 30 x 52μ in size round or ellipsoidal with heavy radially striated shell and containing a 6-hooked hexacanth embryo.	Indistinguishable from those of *T. saginata.*	Ova large averaging 45 x 70μ elliptical, brown in color and provided with an operculum.
Primary host	Man	Man	Man, dog, wolf, bear and other carnivores.
Intermediate host	Normally cattle	Normally hogs	Primary intermediate hosts are minute crustaceans. Secondary intermediate hosts are various fresh water fish.
Cycle in brief	Expelled single proglottids or ova scattered on soil with feces. When taken up by cattle liberated hexacanth embryo bores through intestinal wall into circulation where it is carried to intermuscular connective tissue. In muscle tissue it encysts and becomes a cysticercus.	Terminal proglottids frequently discharged in chains (ova may be expelled singly) taken up by hogs. In alimentary tract of hog, hexacanth embryo bores through intestinal wall and is carried by circulation to intermuscular connective tissue where cysticercus develops in 3 or 4 weeks. When infective cysticerci are taken up by man the worm matures in about 3 months.	Discharged eggs, after period of development in water, liberate ciliated embryo or coracidium which is taken up by crustacean where first stage of larval development occurs. If infected crustacean is eaten by any one of several species of fish the larva works its way out of alimentary tract and lodges in the muscles or viscera becoming infective plerocercoid. Development is completed when plerocercoid is taken up by suitable primary host.
Remarks	Cysts survive 6 weeks in meat refrigerated at 32° F. and about 6 days in meat kept at 15° F. Heating to 159° F. kills cysts in 5 minutes.	Principal danger in case of this species of tapeworm is that autoinfection may occur, i.e., man may serve as the intermediate host. Chief methods of control are through cooking of pork and effective treatment of human cases.	Plerocercoids are killed by cooking fish for 10 minutes.

Diagnostic Characters	*Hymenolepis nana*	*Hymenolepis diminuta*	*Dipylidium caninum*
Common name	Dwarf tapeworm of man	Rat tapeworm	Double-pored tapeworm of dogs and cats.
Attachment hooks	Rostellum armed with single row of hooks.	Rostellum small, unarmed	Retractile rostellum with 3 or 4 rows of easily detached hooks.
Attachment suckers.	4 suckers	4 suckers	4 rounded suckers.

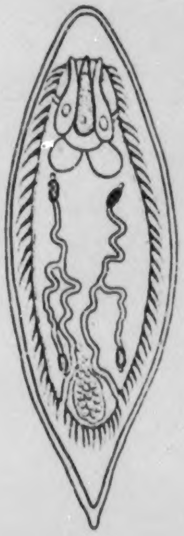
Table 3. A Comparative Table of the Cestodes of Medical Importance—Continued

Diagnostic Characters	*Hymenolepis nana*	*Hymenolepis diminuta*	*Dipylidium caninum*
Length	Approximately 2 inches	8–24 inches	4–16 inches.
Number of proglottids.	100–200	Up to 2,000, occasionally even more, trapezoidal in shape.	Usually 50 or less.
Recognition characters of sexually mature proglottids.	Mature ripe proglottids much broader than long. Three testes present and arranged in a transverse row across the proglottid.	Wide, narrow in anterior-posterior dimensions. Genital pores on one side only. Three testes, two on one side of midventral line and one on the other.	Gravid proglottid markedly constricted at anterior and posterior extremities, in shape of a cucumber or pumpkin seed. Genital pores on each side at center of each bilaterally symmetrical proglottid.
Ova	Eggs elliptical with two membranes, the inner one of which has polar knobs from which thread-like filaments arise. Ova contain hexacanth embryo.	Ova similar to those of *H. nana*, larger, 60 x 80μ with double shell, the inner wall of the outer shell distinctly undulated.	Ova almost sperical 45 x 50μ with shell consisting of two thin layers. Ova contains hexacanth embryo.
Primary host	Generally rats and mice. Most common tapeworm of man in United States.	Generally rats and mice and occasionally man.	Generally dogs and cats. The species is an incidental parasite of man.
Intermediate host.	No intermediate host required. Entire cycle can be and generally is completed in one host.	Various insects including larval fleas and certain species of cereal infesting beetles.	Various insects including principally larval fleas.
Cycle in brief	Embryonated eggs ingested with food or drink or from soiled fingers liberate hexacanth embryo which bores into intestinal mucosa where it transforms into minute cysticercoid in about one week. Cysticercoid drops back into lumen of intestine and develops into mature worm in about 3 weeks.	Expelled ova are ingested by suitable insect intermediate host and develop into infective cysticercoids. When insects containing infective cysticercoids are swallowed they evaginate the scolex, attach and mature.	Expelled ova eaten by suitable insect intermediate host develop into infected cysticercoids. When infected insects are swallowed the cysticercoid evaginates the scolex, attached and matures.
Remarks	Experimentally the cysticercoid will develop in certain beetles and fleas but intermediate host is not essential in life cycle of this parasite.	Man is an incidental host of this parasite. Development takes place in primary host in about 3 weeks.	A large percentage of dogs are infected. Infected animals should be promptly treated. Majority of human infections occur in children.

TREMATODES (flukes)

Various species of flukes parasitize different organs of the human body including the lungs, liver, intestine, and certain blood vessels in the mesenteries and bladder. Because of their location within the human host, adults are rarely eliminated from the body and adult morphology will not, therefore, be considered here. Depending upon the organs in which adults localize their eggs may be recovered from sputum, feces, or urine. Characteristic morphology of trematode ova will be described and illustrated in the section which follows.

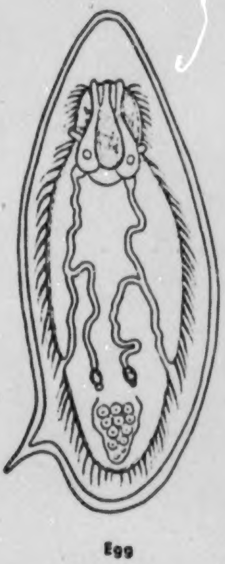
Schistosoma haematobium



Egg

Eggs. Eggs are spindle-shaped, rounded anteriorly and conical at the posterior extremity which is tipped terminally with a delicate, relatively blunt-pointed spine. The egg is nonoperculate, bears a thin transparent single shell, is yellowish-brown in color and normally contains a fully developed ciliated motile miracidium when passed. In older infections many eggs will be in various stages of distintegration.

Schistosoma mansoni

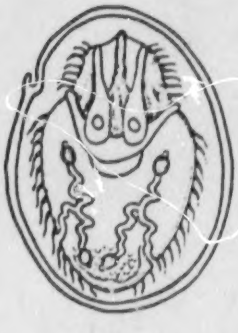


Egg

Eggs. Eggs are narrowly rounded anteriorly and more broadly rounded posteriorly. The most prominent diagnostic character is the presence of a long, sharp, lateral spine in the posterior third of the egg. Eggs are transparent, bear a thin single nonoperculate shell, are yellowish-brown in color, and normally contain a fully developed ciliated motile miracidium. Eggs in various stages of disintegration often occur.

Schistosoma japonicum

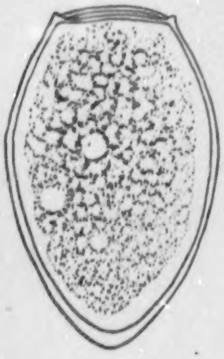
Eggs. Eggs are nearly ovoidal in contour, and only slightly longer than broad. At the side near one end there is a depression from which arises a small incurved spinose process which resembles a small recurved hook. This process can be seen only when the egg is properly oriented. The shell is thin, nonoperculate, single, pale-yellow in color, and normally developed eggs contain a ciliated motile miracidium. Disintegrated eggs often occur.



Egg

Paragonimus westermani

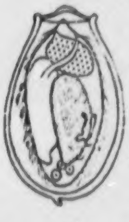
Eggs. Eggs are moderately large, broadly ovoidal, brown in color and capped with a broad, flattened only slightly convex, operculum which is ringed by a raised marginal ridge giving rise to some shouldering. Posteriorly the shell is thicker than in the anterior half. The eggs which are generally passed in the sputum are occasionally swallowed and passed in the feces. Eggs are undeveloped when eliminated from the host and closely packed with yolk cells. There is a broad clear zone between the clustered yolk cells and the shell.



Egg

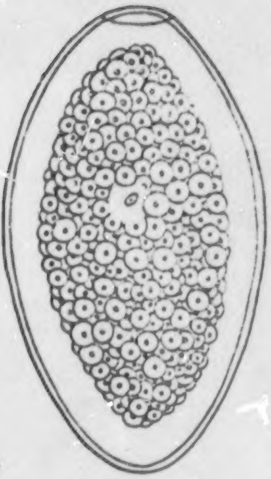
Clonorchis sinensis

Eggs. Eggs of this species are relatively thick-shelled, operculate, and are among the smallest eggs passed by man. They are light brown in color, ovoidal in shape, and the operculum rests within a rimmed depression the margins of which form pronounced shoulders which do not follow the contour of the shell curvature. The posterior end of the egg is thickened and bears a small median protuberance which is oftentimes recurved and comma-shaped in lateral view.



Egg

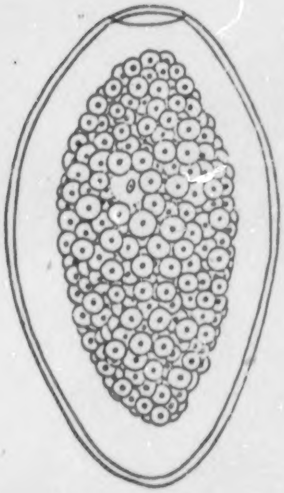
Fasciolopsis buski



Egg

Eggs. Eggs are very large, possess a clear thick shell, are goose egg-shaped, and bear a small convex operculum the contour of which is continuous with the shell. They are undeveloped when passed from the host and packed with yolk cells in which the granules are evenly distributed throughout. There is a broad clear zone between the shell and the mass of yolk cells.

Fasciola hepatica

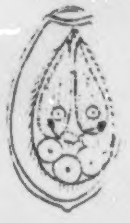


Egg

Eggs. Eggs closely resemble those of *F. buski.* They can be differentiated from the latter species by the distribution of yolk cell granules which in the present species are clustered about the yolk cell nuclei. They also tend to be deep golden-brown in color due to absorption of bile pigment.

Metagonimus yokogawai

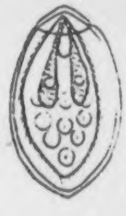
Eggs. Eggs are thick-shelled, yellow-brown in color, operculate and contain a fully developed bilaterally symmetrical miracidium when they are discharged by the host. The operculum is relatively broad and is set on top of a rim and not within it as in the closely related species *C. sinensis.* The shoulder at the point of junction of the operculum with the remainder of the shell is not prominent and the contour of the operculum is almost smoothly continuous with the shell.



Egg

Heterophyes heterophyes

Eggs. Eggs are operculate, very small, brownish in color and contain a well developed bilaterally symmetrical ciliated miracidium. Eggs are difficult to differentiate from those of *C. sinensis* and *M. yokogawai.* Ova can be separated from those of *C. sinensis* by the nature of attachment of the operculum which in this species is set upon and not within a rim.



Egg

Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Men

Diagnostic Characters	*Schistosoma haematobium*	*Schistosoma mansoni*	*Schistosoma japonicum*
Geographical distribution.	Apparently limited to the African continent but widespread there.	Eastern and central Africa. Caribbean area, and Northern South America into Brazil.	Numerous heavily infested endemic areas in China, Japan, and the Philippine Islands.
Morphology......	Male is cylindrical, 12-15 mm. in length and not exceeding 1 mm. in width. Oral and ventral suckers close together and in anterior end of the worm. Alimentary canal forks into two branches just posterior to the ventral sucker but unites again to form a single tube down the center of the posterior half of the body. Females are cylindrical and thread-like, exceeding males in length. Ovary elongate lying anterior to the intestinal tube. Vitelline glands occupy posterior end of the body and lie lateral to the intestine. Uterus consists of a long tube opening to the exterior through genital pore which is immediately posterior to ventral sucker. The female lies within gynecophoric canal of male.	Adults closely resemble those of *S. haematobium*. Male is about 12 mm. long, possesses 6-9 testicular tubules and the short intestinal trunks unite into a single blind pouch which is longer than that of *S. haematobium*. Body of the male is flattened and folded ventrally to form a canal in which female is held by the male. Vitelline glands occupy the posterior two-thirds of the body. Ovary lies in anterior third of the body and at that level the two intestinal trunks converge to form a single blind pouch.	Adults resemble those of the other two important Schistosomes of man. Male is 12-20 mm. long and 0.5 mm. in diameter. Integument is covered with small splines more prominent near the suckers and the gynecophoric canal. There are seven testes. Female about 26 mm. long and integument also covered with spines. Ovary is just a little behind center of body. Vitelline glands limited to lateral margins of posterior quarter of the body. Uterus consists of long straight tube containing up to fifty eggs at one time.
Primary intermediate host(s) in nature.	Man only................	Man only................	Man and other mammals including dogs, cats, horses, cattle, rats, mice, and many others.
Secondary intermediate host(s).	Snails of the genus *Bullinus* commonly and two other genera rarely.	Snails of the genus *Planorbis*.	Snails of the genus *Oncomelania*.

Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Man—Continued

Diagnostic Characters	*Schistosoma haematobium*	*Schistosoma mansoni*	*Schistosoma japonicum*
Life cycle.	Coupled adult worms lodge in the finer veins of the primary host, especially in the bladder and uterus, and the female alone migrates into the finer blood vessels and deposits her eggs in the venules. Eggs by means of their sharp points and lytic secretion force their way through the tissue into the bladder from which they are expelled by the primary host with urine. Occasionally they may be discharged with the feces. Ova contain well developed miracidium and in contact with water the miracidium quickly escape by rupture of the shell after which they seek appropriate snail host. In the latter, two generations of sporocysts are produced. Daughter sporocysts produce the infective fork tailed cercariae (sometimes called furcocercariae) which actively penetrate the skin of man and enter the circulation to lodge in the venules of the bladder, uterus, and sometimes the intestine.	Coupled adult worms lie in the small venules in the wall of the large intestine. Female alone migrates to the end of these venules and packs them with eggs. Eggs by lytic action and by means of their spinous process penetrate out of the small blood vessels and tissues eventually reaching the lumen where they are expelled with feces. Ova that do not reach the intestine soon disintegrate in the blood vessels. On contact with water ciliated miracidium is liberated and seeks out and penetrates appropriate snail. In the snail two generations of sporocysts are produced, the second of which gives rise to the fork tailed cercariae. These escape from the snail and on contact with the skin of man they penetrate and gain entry to the circulatory system and make their way to the small vessels of the large intestine completing the cycle.	Coupled adult worms lie in the smaller venules in the mesentery of the small intestine and frequently within the muscular and mucous layer of that portion of the alimentary tract. Female migrates to the very small branches of the blood vessels and deposits eggs singly packing the venules one at a time. By pressure and the secretion of lytic substances eggs rupture through into the lumen and are expelled with feces. In water, ciliated miracidium ruptures the shell of the egg and swims freely. On contact it invades suitable intermediate snail host. In the snail, sporocysts and daughter sporocysts are produced. Second generation sporocysts produce the cercariae which on contact with suitable primary host enter by active penetration of the skin and reach the circulation through which they are carried to the blood vessels in the region of the small intestine where they grow to maturity thus completing the cycle.
Ova (all very characteristic and readily differentiated).	Large, oval in shape, nonoperculate, measuring about 60 x 160μ tipped at the extreme posterior extremity with a short, sharp spine.	Moderately large, oval in shape, nonoperculate, tipped marginally near the posterior extremity with a sharp lateral spine.	Smaller than the other species of schistosomes, measuring about 45–60μ and bearing near posterior end and to one side of mid-line a wart-like thickening where an abbreviated spine is seen when ova is lying on its more flattened surface.

Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Man—Continued

Diagnostic Characters	*Schistosoma haematobium*	*Schistosoma mansoni*	*Schistosoma japonicum*
Effect on host....	Penetration of the cercariae often produces severe itching and frequently a definite rash persisting for several days. Secondary infection may occur at points of penetration. In later stages of the disease, symptoms are due largely to movement of the eggs in the mucous membranes of the bladder where they may cause inflammation, cause growth to be formed, and lacerate the blood vessels resulting in bloody urine and acute pain on urination. Symptoms in infections of long duration are due largely to movement of ova in the general circulation and their lodgement in various organs, especially the liver.	Disease produced by infection with this species is known as intestinal schistosomiasis. This infection gives rise to dysenteric symptoms, bloody mucous and in older chronic cases to the formation of polypoid growth within the bowel, latter being due to the proliferation of tissue about masses of disintegrated ova which fail to break through into the intestinal lumen.	Causes "Katayama" disease. There are three distinct stages in course of the disease including period of incubation, period of egg laying and period of proliferation and tissue repair, the latter being chronic stage. Latter stage is characterized by great enlargement of the liver and spleen, by intestinal disturbances, thickening of intestinal wall and formation of ulcers and polypoid growths.

Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes

Diagnostic Characters	*Clonorchis sinensis*	*Fasciolopsis buski*	*Paragonimus westermani*
Geographical distribution.	Most important liver fluke of man in parts of China, Japan, Formosa and Indo-China.	Common in India, China, Formosa and Indo-China.	Common infection of man in Asia. A very similar or identical fluke is found in cats, dogs, pigs, and wild carnivores in Africa and North and South America.
Morphology of adult.	Flat, relatively small 10-20 mm long and 2-4 mm wide. Two suckers are present, the oral being larger, and the acetabulum is situated about one-third length of body from anterior end. Testes (two) situated in posterior end, greatly branched.	Largest and best known of the intestinal flukes of man. Worm is leaf-like in appearance, measuring over an inch in length and about a half inch in width. Vental sucker which lies close to the anterior end, is very large and three to four times the size of the oral sucker. Ovary branched at about middle, testes posterior.	Almost round in cross section about 12 mm broad and one-third as wide, reddish brown in color. Surface is covered with large spines of various forms. Caeca elongate, flexious, unbranched. Ovary compact, just posterior to prominent ventral sucker.
Primary host(s)...	Man, cat, dog, mink, pig, and several other mammals.	Man and pigs commonly; dogs occasionally.	Man, possibly the same species also infecting cats, dogs, pigs and various wild carnivores.
Secondary host(s).	Snails of the genus *Bithyina* and certain species of fish.	Snails of the genus *Planorbis* and *Segmentina*.	Snails of the genus *Melania* and certain crustaceans.
Development....	Operculate egg passes with feces from primary host and in water liberates ciliated miracidium which enters suitable snail host. In the snail the miracidium loses its cilia and becomes the sporocyst. Within the sporocyst germinal cells give rise to motile rediae which migrate to the hepatopancreas. In that organ the rediae produce either daughter rediae or cercariae. Cercariae, push their way through the tissues of the snail and swim freely in water. Upon reaching a suitable fish they enter and develop to infective metacercariae in the muscles.	Operculate ova hatch within two or three weeks after discharge and liberated miracidium seeks appropriate snail intermediate host. Development in the snail is similar to that of *C. sinensis*. Liberated cercariae encyst on water chestnut or water caltrop. Human infections result from the habit of cracking water caltrop and water chestnuts with the teeth.	After usual cycle in the snail, knob-tailed cercariae emerge and penetrate the thinner chitin of certain fresh water crabs and crayfish, losing their tails and encysting in the muscles as infective metacercariae. In suitable primary host ingested metacercariae are freed in the small intestine where they penetrate out into the thoracic and abdominal cavities and in about 30 days pass through walls of the lung into the bronchioles.

Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes—Continued

Diagnostic Characters	*Clonorchis sinensis*	*Fasciolopsis buski*	*Paragonimus westermani*
Ova	Small, ovoid, dark-colored, operculate, about 15-30μ, may remain in water as long as five weeks before hatching.	Operculate, large, measuring about 80 x 125μ; yolk granules clustered around yolk cell nuclei.	Relatively large, approximately 60 x 100μ.
Effect on host	Presence of parasites causes marked proliferation of the epithelium and connective tissue of the liver. Heavy infections may cause cirrhosis of the liver and death of the patient.	Causes considerable gastrointestinal disturbance. Heavy infections cause anemia and in very heavy infections, emaciation is a common symptom.	May cause a chronic bronchial cough and bloody sputum suggestive of tuberculosis.

Diagnostic Characters	*Heterophyes heterophyes*	*Metagonimus yokogawai*	*Fasciola hepatica*
Geographical distribution.	Found in Egypt, Palestine, central and south China, Japan, Korea, Formosa and the Philippine Islands.	Common parasite of man in Egypt, Japan, China, Korea and Formosa.	Cosmopolitan in distribution, particularly abundant where domestic sheep and cattle are raised. Found in North and South America, Europe, Asia and Africa.
Morphology of adult.	Very small, approximately 0.5 x 1.5 mm. Oral sucker small, or ventral surface of the body. Ventral sucker about three times as large as oral sucker located adjacent to the ventral sucker and bearing numerous spines.	Very small, about 0.5 x 2.0 mm, roughly triangular in shape about two times as long as wide in side to side dimensions. Anterior extremity narrow and covered with spines. Ventral sucker larger than oral sucker and to one side of median line. Testes lie in posterior end of body, somewhat symmetrical.	Excretory, digestive and reproductive systems well developed. Hermophroditic (both sexes in same organism). Male system consists of two many-branched testes in posterior end of worm connected to seminal receptacle by vas deferens. Seminal fluid passes to region of genital pore through the ejaculatory duct. Female system consists of two ramified ovaries in lower anterior third of organism which connect by means of oviduct to the uterus. Yolk glands on lateral margins empty into uterus through vitelline duct.
Primary host(s)	Man, dogs, cats, rabbits and several other mammals.	Man and several other mammals.	Cattle, sheep, and man. Over 300 human cases are on record.

Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes—Continued

Diagnostic Characters	*Heterophyes heterophyes*	*Metagonimus yokogawai*	*Fasciola hepatica*
Secondary host(s)	Snails of the genus *Pironella* serve as first intermediate hosts and fish as secondary intermediate hosts.	Snails of the genus *Melania* serve as first intermediate hosts and fish as secondary intermediate hosts.	Snails possessing lungs, belonging to the genus *Limnaea*.
Development	Adults live in small intestine of the primary host frequently in large numbers. Ova are completely embryonated when they leave primary host. Miracidia hatch soon after contact with water and seek snails of the genus *Pironella*. After usual development in snail liberated cercariae penetrate beneath scales of certain fish where they encyst as the infective metacercariae.	Infective metacercariae are liberated from their cysts in the duodenum where they burrow into the intestinal mucosa and grow to maturity and deposit eggs. The eggs break out into the lumen and pass out with the feces. They may also break out of intestinal wall into the general circulation.	Poorly developed eggs excreted in feces, develop in water and liberate free swimming miracidium which penetrates snail and lodges in lung tissue where it transforms into the sporocyst. Within sporocyst rediae are produced and they migrate to the hepato-pancreas and produce either daughter rediae or cercariae. The single tailed cercariae escape from the snail, and encyst on vegetation as metacercariae. On being taken by definitive host, they are liberated in the intestine, grow to maturity and then by way of the bile ducts they migrate to the liver.
Ova	Operculate, small, brownish in color, containing well developed ciliated miracidium. Difficult to distinguish from those of *C. sinensis* and *M. yokogawai*.	Operculate, small, brownish in color, containing well developed ciliated miracidium. Difficult to differentiate from those of *C. sinensis* and *H. heterophyes*.	One of the largest of helminth eggs, ovoid in shape and about 80 x 150μ yellowish-brown in color, operculated. Eggs immature when discharged, development taking place in water.
Effect on host	Of little pathogenic importance in either man or animals.	In very heavy infections persistent intestinal disturbances and diarrhea are present. Ova deposited into the general circulation reach the heart and central nervous system, producing cardiac and nervous disturbances.	Moderate to heavy infestations in man give rise to severe symptoms. Patient loses weight and becomes weak and anemic. In animals there are marked liver changes including fibrosis in well established infections. The disease in animals is known as "liver rot".

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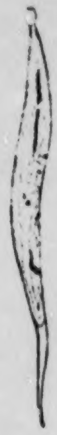
NEMATODES (roundworms)

Roundworms constitute the most common parasites of man throughout the world. In tropical and semi-tropical regions infection with one or more nematodes is acquired very early in childhood and persists throughout life. Among poorly sanitated native populations in the tropics these infections are universal. Roundworm infections are not uncommonly encountered among our troops stationed in regions of the world where a high reservoir of infection exists in the indigenous population.

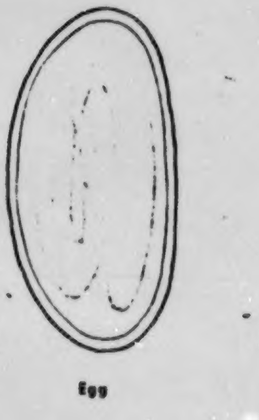
Laboratory diagnosis of roundworm infection is based upon recovery and identification of eggs, larvae, or adults depending upon the particular species harbored by the patient and the stage of development in which infective stages are eliminated. In freshly passed stools adults are usually intact since their bodies are fairly rigid, unsegmented, and the outer covering or cuticula is quite resistant to bacterial action and decay. In order to observe morphological characters the worms should be processed and mounted preparations made according to techniques described elsewhere in this manual.

Enterobius vermicularis (pin-worm)



Adult Female

Adults. Adults are ivory white in color, rigid, bristle-like, with the posterior third of the body attenuated and terminating in a fine rather sharp point. The organism does not exceed one-half inch in length. The head is slightly expanded and bears three labia in a triangular pattern as observed in direct anterior view. The long muscular esophagus terminates in a distinct bulb.

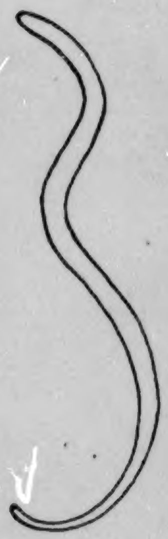


Egg

Eggs. Eggs are flat on one side and broadly rounded on the opposite side, their contour being suggestive of loaves of bread. The shell is moderately thick and consists of three layers, a thick outer albuminous layer, an inner thinner hyaline layer and a membrane which immediately surrounds the embryo. The shell is clear and colorless and eggs normally contain a fully developed motile larva when passed. Eggs are encountered infrequently in routine examinations but in suspected infections they are readily recovered by use of any of several special techniques described elsewhere in this manual.

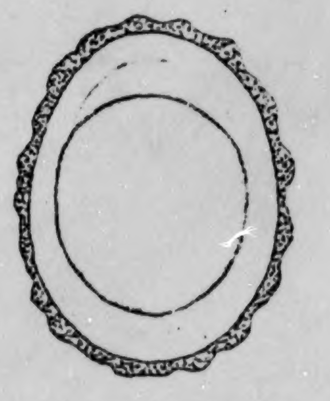
Ascaris lumbricoides (large roundworm of man)

Adult. This is the largest roundworm which infects man. Fully mature females may attain a length of eight inches. When freshly passed they are light flesh colored in appearance and quite relaxed presenting much the appearance of an earthworm. The anterior extremity is blunt-pointed and the organs of attachment consist of three fleshy lobes arranged in a triangular pattern when observed in end view. The posterior extremity is tapered and more finely pointed. Males are about half as large as females and the posterior extremity is re-curved sharply and bears two spicules on the inner margin of the curvature. Spontaneous elimination of adult worms is not uncommon. In children these may be regurgitated with vomitus or the worms may actively migrate from the stomach through the esophagus and creep out the mouth or nostrils. Any heavy bodied roundworm in excess of a few inches in length which is eliminated by humans can be stated with certainty to be either *Ascaris lumbricoides* or the closely related and morphologically indistinguishable *Toxocara canis*, normally found in dogs but which may also infect man, particularly children.



Adult Female

Eggs. Fertilized ova are normally broadly ovoid, and the outer covering or cortex consists of a thick lamellated protective covering beneath which is a smooth surfaced hyaline moderately thick shell. The eggs are unsegmented when passed, filled with highly granular yolk material, and dark golden-brown in color due to marked absorption of bile pigments.



Normal Corticated Fertilized Ova

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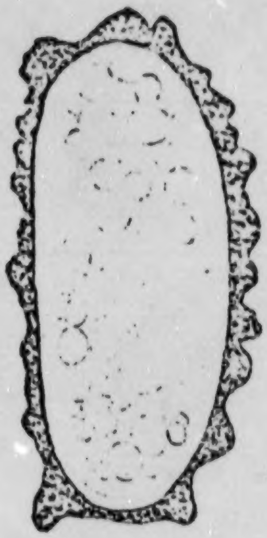
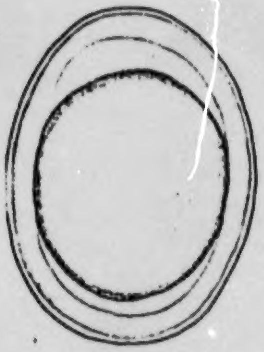


Corticated Unfertilized Ova

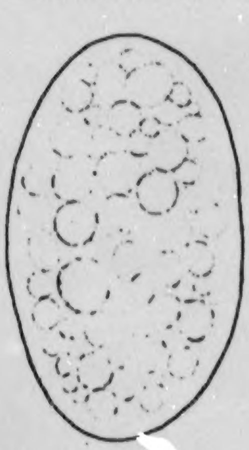
Unfertilized eggs are almost twice as long and somewhat more narrow than fertilized eggs and the outer protective covering and inner shell are thinner. Egg contents consist of large irregular granules and clear non-granular areas. Infertile eggs are encountered in about forty percent of infections and about fifteen percent of all eggs are infertile.



Decorticated Fertilized Ova

The outer, easily broken lamillated albuminous covering is at times sluffed off and absent. This is particularly true when examinations are preceded by agitation as, for example, concentration techniques employing centrifugation.

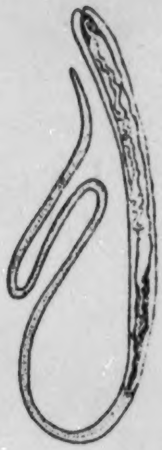
It follows from the above discussion that four types of eggs may be encountered in routine examinations: fertilized corticated, unfertilized corticated, fertilized decorticated, and unfertilized decorticated.



Decorticated Unfert[illegible]d Ova

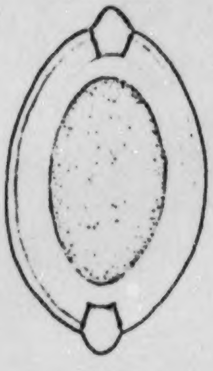
Trichuris trichiura (whip-worm)

Adult. Grossly the adults are whip-like in contour, hence the name "whip-worm." Anteriorly more than half the length of the worm consists of a thread-like filament which broadens into the fleshy portion of the body toward the posterior extremity.



Adult Female

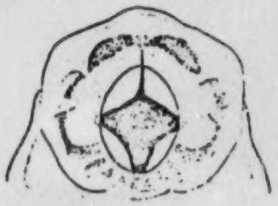
Eggs. The eggs are very characteristically barrel-shaped, widest at the middle and tapering smoothly toward the rounded polar prominences, which are sometimes referred to as knobs. The eggs are unembryonated when oviposited and filled with a homogeneous mass of yolk granules which is slightly withdrawn from the inner shell. Eggs bear two prominent shells the outermost of which is thick and bile-stained and the inner of which is hyaline. The mucoid plugs which give rise to the polar prominences penetrate through the outer shell to the margin of the inner shell.



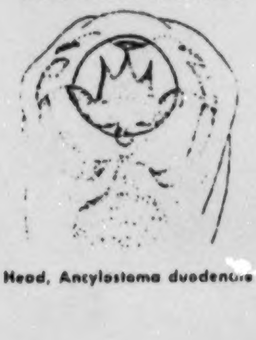
Ova

Necator americanus Ancylostoma duodenale (hookworms)

Adult. Mature worms are a little less than an inch in length. The two common species which infect man can be differentiated from each other on the basis of the number, type and arrangement of teeth in the buccal cavity, the position of the genital pores in the females, and the number and arrangement of supporting rays and spines in the caudal bursa.



Head, Necator americanus



Head, Ancylostoma duoden

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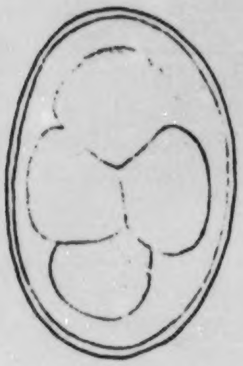
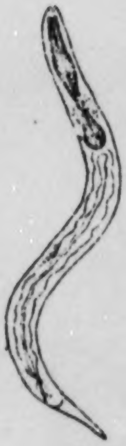


Egg

Eggs. The eggs of all species of hookworm of man and lower animals are identical and cannot be differentiated. Hence they are referred to as hookworm ova without reference to species designation. The eggs are ovoidal in shape, glass clear, uncolored, and have smoothly rounded ends. They are approximately one and one-half times as long as wide. In fresh fecal specimens ova will usually contain embryos in the four to eight cell stage of development. In constipated stools or in situations where stool examination is delayed following passage fully embryonated eggs which contain a motile larva may be seen. These eggs may hatch in the stool specimen releasing free larvae which are easily confused with those of *Strongyloides stercoralis.*

Strongyloides stercoralis



Larva

Larvae. Eggs are not normally passed in the stools of humans harboring this species of roundworm. If on occasion, eggs are eliminated by the host they are morphologically indistinguishable from those of hookworm and would, therefore, be likely designated as such. The eggs usually hatch in the intestine liberating the first stage or rhabditiform larva. As stated above under certain conditions in hookworm infection motile larvae may be encountered in fecal examinations. The larvae of the present species can be differentiated from those of hookworm by the fact that in *Strongyloides* the esophagus is much longer and the larvae are considerably larger.

Table 6. A Comparative Table of the Two Common Human Hookworms

Diagnostic Characters	*Ancylostoma duodenale*	*Necator americanus*
Common Name.....	Old world hookworm..................	American hookworm—New world hookworm.
Geographical distribution	Europe, Southern Africa, India, the Far East, the Pacific Islands, Northeastern Australia, and a limited portion of the east central coast of South America.	Warmer regions of the entire western hemisphere, the southern half of the continent of Africa, India, the Far East, Northeastern Australia and the islands of the Central Pacific.
Morphology........	Males and females of almost equal size, measuring 8–13 mm in length. Buccal cavity broadly ovoid, bearing two pair of prominent dorsal teeth and a pair of less prominent ventral cutting plates. The posterior extremity of the female is bluntly pointed while that of the male possesses a broad fan-shaped dorsal bursa within which lie two bristle-like spicules. The latter trail free behind the posterior margin of the bursa. The vulva of the female is posterior to the middle of the body.	Males and females of almost equal size, species smaller and more slender than *A. duodenale*, measuring 7–11 mm in length. Buccal cavity broadly ovoid, small, armed with a pair of fairly prominent cutting plates on the dorsal aspect and a corresponding pair of markedly less prominent cutting plates on the ventral aspect. Posterior extremity of male roughly similar to the male of *A. duodenale*, important differences occurring in number and arrangements of dorsal rays and in the appearance of the copulatory spicules. In the present species the latter are fused terminally and end in a backwardly directed barb. The vulva of the female is at about the middle or or anterior to the middle of the body.
Egg production.....	10,000–20,000 eggs daily.	5,000–10,000 eggs daily.
Ova...............	Ovoidal in shape with longitudinal axis only slightly longer than the transverse axis. The single shell is thin and transparent, and in normal stools eggs are usually in the 4–8 celled stage of segmentation when passed.	Similar to *A. duodenale*.
Cycle in brief.......	Under optimum environmental conditions eggs hatch in less than 24 hours liberating a rhabditiform larva. Within about a week the rhabditiform metamorphoses into the infective filariform larva. Larvae normally enter the host by active penetration of the skin whence they make their way to the lungs via the circulatory system. In the lungs they burrow into the air spaces making their way to the small intestine via the pulmonary and alimentary tracts. Following a period of development in the intestinal submucosa the developing worms attach to the intestinal mucosa and complete the cycle in about 6 weeks.	Similar to *A. duodenale*.

Table 7. A Comparative Table of Medically Important Intestinal Nematodes (Other Than Hookworms and Strongyloides Sp.)

Diagnostic Characters	*Ascaris lumbricoides*	*Trichuris trichiura*	*Enterobius vermicularis*
Common name	Giant intestinal roundworm	Whipworm	Pinworm or seatworm.
Geographical distribution.	Cosmopolitan with greatest incidence in moist, warm regions, especially those where human excrement is used as fertilizer.	Cosmopolitan. More common in the warm moist regions of the world, particularly in the tropics.	Cosmopolitan, much more common in children than in adults, infection not dependent upon climatic conditions, but upon personal habits of individuals.
Morphology	Largest nematode infecting man, mature females sometimes attaining a length of over 40 cm, males considerably smaller. Attachment organ consisting of a dorsal and two ventral lips, the three lips of about equal size forming the apices of a roughly equilateral triangle. Freshly passed worms "fleshy" in color and exhibiting motility. Male readily distinguished from female by marked posterior ventrad curvature.	Males and females of approximately the same size, measuring 30–50 mm in length. Body whip-like anterior portion filamentous and the posterior portion fleshy. Posterior portion of male markedly recurved ventrad, often forming a complete circle. Spicule of male prominent, sheathed. For attachment the anterior thread-like porcion of the worm is intertwined into the intestinal submucosa.	Attachment organ consisting of three retractile labia forming a triangle in "in face" view. Females approximately 10 mm in length; males about half as large. Posterior extremity of female marked by attenuation terminating in "pin" point. Male readily distinguished from female by marked posterior ventrad curvature.
Egg production	Capacity of genital tubules around 27 million ova, with estimated egg production of 200,000 daily.	About 2,000 eggs per day	Capacity of genital tubule approximately 20,000 ova. Daily egg production variable.
Ova	Variable in size and shape depending upon fertility and presence or absence of albuminous lamellated cortical covering. Fertilized ova broadly ovoid, brown in color, containing circular mass of granular yolk material enclosed in a thick, smooth inner shell, and surrounded by albuminous covering referred to above.	The ova are barrel-shaped and possess transparent polar prominences. They are brown in color and have two shells, an inner thin shell in turn enveloped by an outer thick resistant covering.	Shell moderately flat on one side, broadly oval on the other, clear, hyaline, consisting of an outer rather thin albuminous layer and a thin inner membrane. Ova almost fully embryonated when passed.

Table 7. A Comparative Table of Medically Important Intestinal Nematodes (Other Than Hookworms and Strongyloides Sp.)—Con.

Diagnostic Characters	*Ascaris lumbricoides*	*Trichuris trichiura*	*Enterobius vermicularis*
Cycle in brief	Direct. Embryonation of ova takes place under optimum conditions of temperature, moisture and oxygen within 9 days following passage from host. Under adverse conditions eggs may remain viable for several years. Infection occurs through ingestion of ova containing fully developed rhabditoid larvae. In duodenum the egg hatches, liberating larva which penetrates intestinal wall and by way of the circulatory system reaches the lungs. Following period of development immature worm penetrates out of alveoli and follows air passages to epiglottis from which it follows alimentary tract to small intestine where it reaches maturity in about two months.	Cycle is direct. Embryonation of ova takes place entirely outside of host and requires at least 21 days under optimum environmental conditions. On ingestion larvae are liberated in the small intestine where they undergo partial development within the submucosa. They gradually migrate to the caecum and appendix where they attach and grow to maturity in about three months.	Gravid females migrate to perianal region to deposit ova. Cycle is direct, and complete embryonation takes place within a few hours after ova pass from host. Following ingestion ova pass to duodenum where they hatch and set rhabditoid larva free in lumen. After undergoing some development in duodenum, immature larvae pass to large intestine where they reach maturity and complete the cycle in about three weeks.
Remarks	Should be regarded as one of the most dangerous helminth infections of man.	From a therapeutic standpoint this is the most difficult helminth to dislodge from the intestine.	Most common nematode infection of man in the United States.

PSEUDOPARASITES AND ARTIFACTS

A guide to the identification of objects which may be encountered in routine fecal examinations would be most incomplete if a strong word of caution concerning objects which may fool the unwary or the inexperienced technician was excluded from the discussion. These objects are generally referred to as "pseudoparasites" or "artifacts." Pseudoparasites can be defined as objects which resemble parasites or ova, but are either not parasites at all or are not parasitic in man. Their accurate identification is of much less importance than their separation from objects which are diagnostic of actual intestinal parasitic infections. Unfortunately, there is no simple yardstick which can be applied as a criterion for determining whether a given object is truly a parasite or whether it is in actuality any one of the myriad of objects which may be present in a stool specimen at the time of examination. Some of the objects more commonly encountered in fecal examinations, which may be confused with true parasites, are illustrated in accompanying figure 3-1.

In distinguishing between objects which are parasites and objects which are not, factors

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which must be considered include the conditions under which the specimen may have been collected, the age of the specimen, and technique utilized to prepare the specimen for microscopic observation. It must be emphasized that the error of identification can extend in either direction; that is, objects which are not parasites may be wrongly identified as such, and true parasites may be misidentified as pseudoparasites or artifacts.

Objects which may be incorrectly identified as parasites fall into several broad categories. These include cellular elements which are normally shed into the intestine from various points in the digestive tract, remnants of debris originating from solids ingested by the patient in food or drink, yeasts and fungi which are normal inhabitants of the intestinal tract, and contaminants introduced into the specimen after it has been passed by the patient.

Cellular Elements from the Intestinal Tract

Various cellular elements originating from the intestinal tract may be present and these often closely resemble intestinal protozoan cysts and trophozoites. In certain situations, such as in dysenteric stools or stools from patients suffering from chronic disease of the intestinal tract, these cells may be found in large numbers. Cells commonly encountered are columnar epithelial cells from the gut, squamous cells from the anal mucosa and leukocytes including large endothelial macrophages. Careful observation of structural characteristics of the nuclei, their relative size in relation to the cellular cytoplasm, and the nature of cytoplasmic inclusions are the most diagnostic criteria for distinguishing these cells from protozoans.

In fresh preparations, leukocytes may exhibit pseudopodial activity and motility. Careful observation under varying intensities of light will usually reveal the presence of uniform-sized highly refractile granules easily distinguishable from characteristic protozoal inclusions. In hematoxylin stained preparations, polymorphonuclear leukocytes may exhibit nuclei closely approximating those present in amebic cysts. On close study, it will generally be seen that these nuclei are larger in relation to the cytoplasm, than in amebic cysts. They also tend to be unequal in size, irregular in outline, and stain more intensely; and they fail to show uniformity of internal morphology corresponding to karyosome bodies and even distribution of inner peripheral chromatin.

Large endothelial macrophages containing ingested red cells may be mistaken for the trophozoites of *E. histolytica*. In the nucleus the absence of a structure corresponding to the karyosome body, and the presence of fine, rather evenly distributed network of chromatin interspersed with a scattering of relatively large deeply staining particles scattered throughout, usually facilitate recognition. Columnar epithelial and squamous cells are flat and will be noted to lack the dimensional qualities of protozoans as depth of focus is carefully manipulated.

Substances Ingested with Food or Drink

Numerous substances which may be ingested remain sufficiently unaltered in their journey through the intestinal tract that they may be eliminated in various sizes, and in such configurations as to be easily confused with whole parasites, portions thereof, or stages in their development. Among these are cysts of normally free living protozoans, ova or adults of free living nematodes, ova, whole larvae, body parts or whole adult insects, the indigestable cell walls or other portions of plant and animal tissues, and pollen grains.

Free living protozoa that live in fecal material are known as coprozoic species. Cysts of these normally free living forms may pass through the body unchanged. Or, they may be ingested, and after passing through the body, excyst when they are exposed to room temperature. In this manner, both cysts and trophozoites of protozoans which are foreign to the intestine of man may be recovered in fecal examinations.

Soil nematodes are common parasites of the roots of plants. In the case of vegetables which are eaten raw, the possibility of ingestion of the worms or their ova and their ulti-

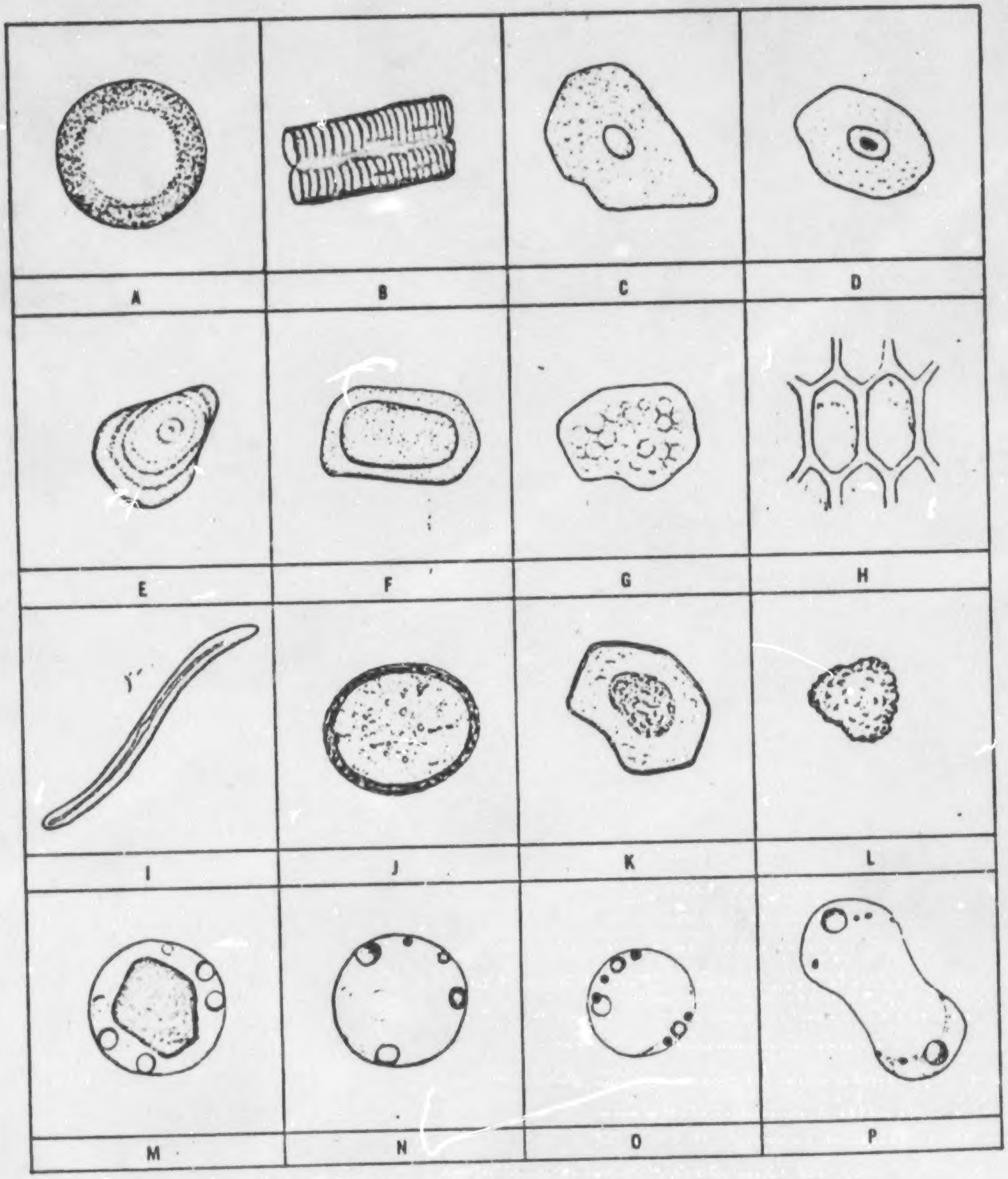

Figure 3–1. Pseudoparasites and Artifacts Commonly Mistaken for Intestinal Parasites.

A, oil globule. B, partially digested muscle fiber. C and D, epithelial cells. E thru H, vegetable cells. I, plant hair. J thru L, pollen grains. M thru P, Blastocystis sp.

mate recovery in fecal examinations is apparent. While it is known that the ingestion of the ova of certain species of Diptera may give rise to intestinal myiasis, the recovery of either dead or viable dipterous larvae must be carefully interpreted in the light of all possible factors before it can be concluded that larvae present are responsible for this type of parasitic infection. Certain species of oviporous Diptera seek feces upon which to deposit their living young and specimens left unprotected against flies following passage may immediately have maggots deposited upon them. Unprotected fecal specimens may serve as habitats of choice for oviposition by oviporous species. Under optimal conditions of temperature these soon hatch and with resultant maggot infestation of specimens. Since no adult arthropods are known to be associated with parasitism in man the recovery of portions of, or whole insects is always due to accidental ingestion and subsequent expulsion rather than to parasitism.

Vegetable cells, starch granules, and animal cells from food are of many shapes and sizes and may resemble cysts, ova, or nematodes. Starch granules may be quite spherical, and if undigested, they are composed of successive concentric rings of gray homogeneous material. Potato starch cells often occur in clumps of sack-like, fairly uniform aggregates suggestive of egg packets of *D. caninum.* In iodine stained preparations, undigested starch will stain blue while partially digested starch particles assume a pinkish hue.

The undigested, individual spindle-shaped vesicles of citrus fruit such as oranges and graefruit are worm-like in outline and may easily be mistaken for adult pinworm. Close examination of the interiors reveals that these sacs are hollow and structureless. Fibrous plant hairs and lint fragments may also be mistaken for nematodes. Ingested pollen grains may be mistaken for helminth ova. Their lack of characteristic yolk granules or developing embryos, and the absence of a clearly delineated shell serve to distinguish them from ova.

Normal Non-Parasitic Inhabitants of the Intestinal Tract

Perhaps the greatest sources of confusion and error in the identification of objects seen in microscopic examination of feces are yeasts and fungi. These are normal inhabitants of the intestinal tract. *Blastocystis hominis,* a harmless intestinal commensal is the species most easily mistaken for a protozoan cyst. It is spherical in outline and its gradations in size correspond to those of the larger amebae of man. It is characterized by a spherical central mass which is devoid of morphological structures, and the outer wall within which the nuclei are contained is relatively thick and surrounded by a thin capsule. In iodine stained preparations, the central mass does not stain but the thickened outer zone takes on a yellowish-brown color. Within this latter area the nuclei, usually about 3–7 in number, are evenly distributed and very darkly stained. Most of the other species of yeast cells encountered are ovoid in shape and they too, fall within the range of the various protozoan cysts. Yeast cell nuclei are solid, they lack internal morphological structure, and stain blue-black with hematoxylin stains. The best criterion for separating yeasts from parasitic protozoa is the examination of a series of these cells for budding forms.

Contaminants

The necessity for collecting fecal specimens in clean, covered disposable containers cannot be overemphasized. Glassware in which specimens are processed and mixing devices must be scrupulously clean and free of all extraneous matter. Equally important precautions apply to the care which must be exercised in the selection of diluents to be added to specimens during examination. Failure to exercise these precautions may result in the introduction of all manner of objects which may lead to erroneous embarrassing laboratory errors. Objects which may be negligently introduced include, but are by no means limited to, cysts and trophozoites of free living amoebae, free living ciliates, yeasts, pollen, and fly maggots.

PART TWO

EXAMINATION OF BLOOD

Chapter 4

MALARIA

Malarial parasites are most numerous in the blood and most readily found just before the beginning of a chill. The most satisfactory procedure for the definitive diagnosis of malaria is the use of a thick and thin blood film on the same slide. The thick film serves to concentrate the parasites. Prior to staining, the thick film is rendered transparent by lysing the red cells and freeing the hemoglobin. Species identification may not be possible from the thick film alone. If this film is positive for malaria parasites, it may be necessary to turn to the thin film for species identification.

PREPARATION OF BLOOD FILMS

The slide must be chemically clean in order to assure uniform blood films which will adhere to the slide. Cleanse the fingertip or ear lobe with an alcohol sponge, dry with a sterile piece of cotton or gauze, and puncture the skin with a sharp lancet. The thin film is prepared exactly the same way as for routine differential blood count. The film is prepared so as to occupy two-thirds of the length of the slide. The remaining clean end of the slide is used to prepare the thick film. To prepare a thick film, touch the surface of the slide to the drop of blood on the finger or ear lobe, and move the slide in a circular manner until a film about the size of a dime is obtained; this film should be of such thickness that ordinary printing can barely be read through it. The proper thickness is critical since films which are too thin will contain fewer parasites and those which are too thick tend to crack on drying. The slide may be identified by writing on the thick end of the thin smear with an ordinary pencil. The slides prepared in the above manner are placed in a slide box to protect them from dust and insects until they are stained. The slide is ready for staining after the thick film has dried. It is desirable to stain the slides within a day after preparing them to get maximum staining quality. This is particularly important for the thick film. The latter tends to harden with age and becomes difficult to dehemoglobinize.

STAINING OF BLOOD FILMS

Of the several stains which can be used for demonstrating malaria parasites, Giemsa's stains the organisms most sharply and deeply and such preparations resist fading for many years. The principal disadvantage of this procedure is that it is considerably more time consuming than the other methods. Wright's stain takes only a few minutes and is, therefore, very good for rapid diagnosis. The JSB Technique is rapid and parasites stand out very clearly, but preparations stained by this method fade rapidly. Field's stain is rapid and the same stain can be used repeatedly. This technique is not widely used but is excellent for rapid diagnostic work. With this method, if the blood is anemic, staining may have to be prolonged up to 10 seconds and the reticular substance of immature erythrocytes is stained blue and easily confused with malaria organisms. This method was developed and used widely during

World War II and proved very useful where large numbers of blood films were being prepared for microscopic examination. Preparations stained in this manner also fade rapidly and the method is of no value where permanent preparations are desired. All four staining techniques herein described can be utilized for both thick and thin blood smears.

Giemsa's Stain:

Materials and Equipment:

Giemsa's Stain (29)
Staining jars
Methyl alcohol
Slides with thick and thin films
Buffered water (pH 7.0) (16)

Technique:

1. Add 1 part of Giemsa's stain to approximately 50 parts of distilled water. (The optimum dilution will vary with each batch of stain.)
2. Fix the thin film only for 1 minute with methyl alcohol. Do not let alcohol touch the thick film.
3. Allow the slide to dry and then place entire slide in staining solution for 45 minutes.
4. Remove and dip 3 times in buffered water (pH 7.0).
5. Immerse thick portion only in buffered water for 3 minutes.
6. Remove slide and drain dry. Do not blot.

Field's Stain for Thick Films:

Materials and Equipment:

Field's stain, solution A and B (25, 26)
Staining jars
Slides with thick blood films

Technique:

1. Dip thick film for 1-5 seconds in solution A.
2. Rinse by dipping in water until stain ceases to flow from the film.
3. Dip for 1-5 seconds in solution B.
4. Rinse by dipping gently in water for 2-3 seconds.
5. Drain dry.

JSB Stain (Jaswant-Singh-Bhattacherji):

Materials and Equipment:

JSB stain solutions A and B (44, 45)
Staining jars
Slides with thick and thin blood films

Technique:

1. Thin films.
   a. Fix smear in methyl alcohol for 1 minute and dry.
   b. Place in solution A for 30 seconds.
   c. Wash in water (pH 6.2-6.6)
   d. Place in solution B for 1 second.
   e. Wash in same water as step c.
   f. Replace in solution A for 30 seconds.
   g. Wash in same water as in step c until the smear has a pink background.
2. Thick films.
   a. Lake the thick film in water until it has a gray appearance, and dry.
   b. Place in solution A for 10 seconds.
   c. Wash in water (pH 6.2-6.6) for 2 seconds.
   d. Place in solution B for 1 second.
   e. Wash in same water as in step c until the smear gives a pink background.

Wright's Stain:

Materials and Equipment:

Wright's stain (72)
Buffered water (pH 7.0-7.2) (16)

Technique:

1. Lake the thick film by immersing thick film only in buffered water (pH 7.0-7.2) for 10 minutes.
2. Remove and rinse thick film by dipping in fresh buffered water.
3. Allow film to dry thoroughly. Do not blot.
4. Cover entire slide (thick and thin film) with Wright's stain for 1 minute. (This fixes the film.)
5. Dilute by adding an equal quantity of buffered water and let it stain for 4-6 minutes.
6. Carefully float off metallic scum and rinse by dipping in buffered water and allow to drain dry. Do not blot.

Note: For thin films only, start with step 4.

Table 1. A Comparative Table of the Three Common Species of Human Malaria

Stages of Development	*Plasmodium vivax*	*Plasmodium malariae*	*Plasmodium falciparum*
Young trophozoite.	Ring form usually with single large chromatin dot. Cytoplasmic circle thin, usually large, pale blue in color.	Ring form with heavy single chromatin dot. Cytoplasmic circle small, thick, and compact.	Ring form with single or double small chromatin dots. Multiple rings in one cell common. Marginal or "applique" rings occasionally occur and are characteristic when present. Rings displaying two chromatin dots common.
Growing trophozoite.	Contour irregular with several finger-like pseudopods. Fine yellowish brown pigment granules in cytoplasm. Chromatin dots single and compact or thread-like, often surrounded by large vocuole.	Cytoplasm compact, frequently in form of a parallel sided or trapezoidal-shaped band across the center of the red cell. Coarse dark brown or black pigment granules often peripherally arranged on margin to trophozoite.	This is usually oldest asexual stage seen in peripheral circulation. It remains in ring form but chromatin and cytoplasm increase in size. Chromatin granules few in number and yellowish in color.
Large trophozoite.	Parasite practically fills enlarged cell. Chromatin in one abundant loose or compact mass. Fine brown hemazion pigment granules abundant.	Parasite fills or almost fills normal sized red blood cell. Pigment granules large, dark, generally peripherally arranged. Cytoplasm dense, dark blue, often in form of a broad band.	Stage seldom seen in peripheral blood. Very small, solid, with one mass of chromatin. Cytoplasm compact, light blue. Very dark pigment scattered throughout cytoplasm or collected in one small dense block.
Presegmenting schizont.	Chromatin divided into two or more irregular masses. Pigment generally clumped into several large particles. Cytoplasm showing various degrees of separation into strands.	Chromatin divided into a few masses. Cytoplasm dense, dark blue in color. Pigment black, evenly distributed throughout parasite.	Rarely seen in peripheral blood. When found parasite is small and pigment is usually clumped in one small dark mass.
Mature schizont or segmenter.	Divided into 12-24 discrete merozoites with oval nuclei surrounded by a similarly shaped mass of cytoplasm. Merozoites arranged either irregularly or in two rings with one or two eccentric clumps or hemozoin granules.	Divided into 6-12 discrete merozoites (usually about 8) generally in a single circle and surrounding a large clump of hemozoin granules. This is called the "rosette" or "daisy" form.	Rarely seen in peripheral blood. Divided into 8-32 very small discrete merozoites. Fills about two-thirds of normal sized red blood cells.

Table 1. A Comparative Table of the Three Common Species of Human Malaria—Continued

Stages of Development	*Plasmodium vivax*	*Plasmodium malariae*	*Plasmodium falciparum*
Macrogametocyte	Outline circular or ovoid and regular. Cytoplasm homogenous, without vacuoles. Chromatin dot single, small, compact, dark red in color, and excentric in position. Abundant dark pigment granules scattered throughout cytoplasm.	Outline circular or ovoid and regular. Cytoplasm dense, dark blue, containing abundant coarse dark pigment. Chromatin the same as in *P. vivax*.	Long, slender, sausage-shaped or crescentic with a concentrated mass of dark pigment near center surrounding a dark red chromatin mass.
Microgametocyte	Usually circular in outline, when fully mature about size of normal red cell. Pigment yellowish-brown abundant throughout cytoplasm. Cytoplasm stains very light blue. Chromatin mass large, diffuse, light red in color at or near center of microgametocyte and often surrounded by a clear vesicular area.	Same as in *P. vivax*, but smaller. When fully grown usually does not fill normal sized red blood cell.	Cytoplasm generally paler than in macrogametocyte. Parasite broader, shorter, and with more rounded ends than macrogametocyte Granules of pigment surrounding light staining granules or threads of chromatin throughout central half or more of parasite.
Length of asexual cycle.	48 hours	72 hours	36–48 hours.
Stages in peripheral blood.	All	All	Usually ring form trophozoites and gametocytes only. Other stages rarely found except in severe cases.
Time of appearance of gametocytes.	Early, shortly after trophozoites.	Late—Usually 30 days after trophozoites.	Moderately late—about 10 days after trophozoites.
Incubation period in man.	12–17 days, average 14 days.	21–35 days, average 28 days.	8–14 days, average 12 days.
Period covered by recurrences.	2–3 years	7–10 years	2–6 months.
Infected red cell	Enlarged, pale, displaying Schuffner's dots.	Normal in size, lacking any characteristic stippling.	Usually normal in size. Maurer's dots sometimes present.
Remarks	More developmental stages likely to be observed in one blood film than in any other species.	Parasites compact and hence appear darkly stained.	Parasites usually much more numerous than in other species. Growth of asexual forms other than rings generally takes place in internal organs.

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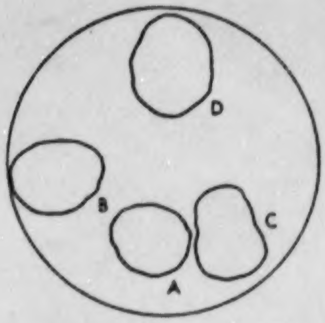
PLASMODIUM VIVAX

(Tertian Malaria)

Color Plates

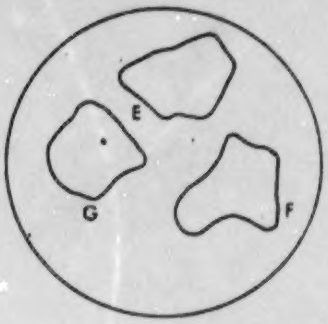
4-5

PLASMODIUM VIVAX (Tertian Malaria) GIEMSA STAIN (x 2000)



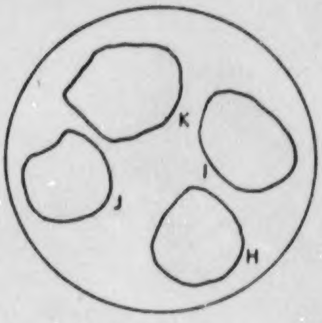
I. Young Trophozoites

A and B—Young ring forms.
C and D—Half-grown trophozoites.



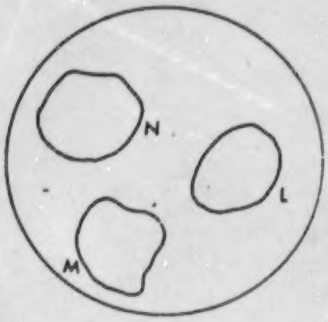
II. Old Trophozoites

E, F, and G—Trophozoites with nuclear chromatin ready to subdivide.



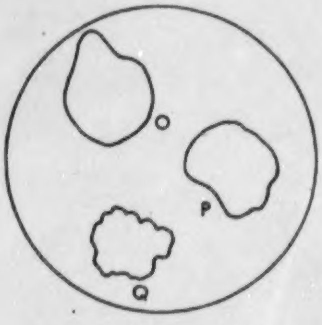
III. Young Schizonts

H and I—Young Schizonts showing first division of nuclear chromatin.
J and K—Older schizonts showing from four to many subdivisions of nuclear chromatin.



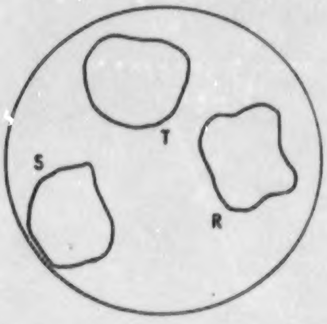
IV. Half-grown Schizonts

L, M, and N—Schizonts showing seven, eight, and ten nuclear subdivisions.



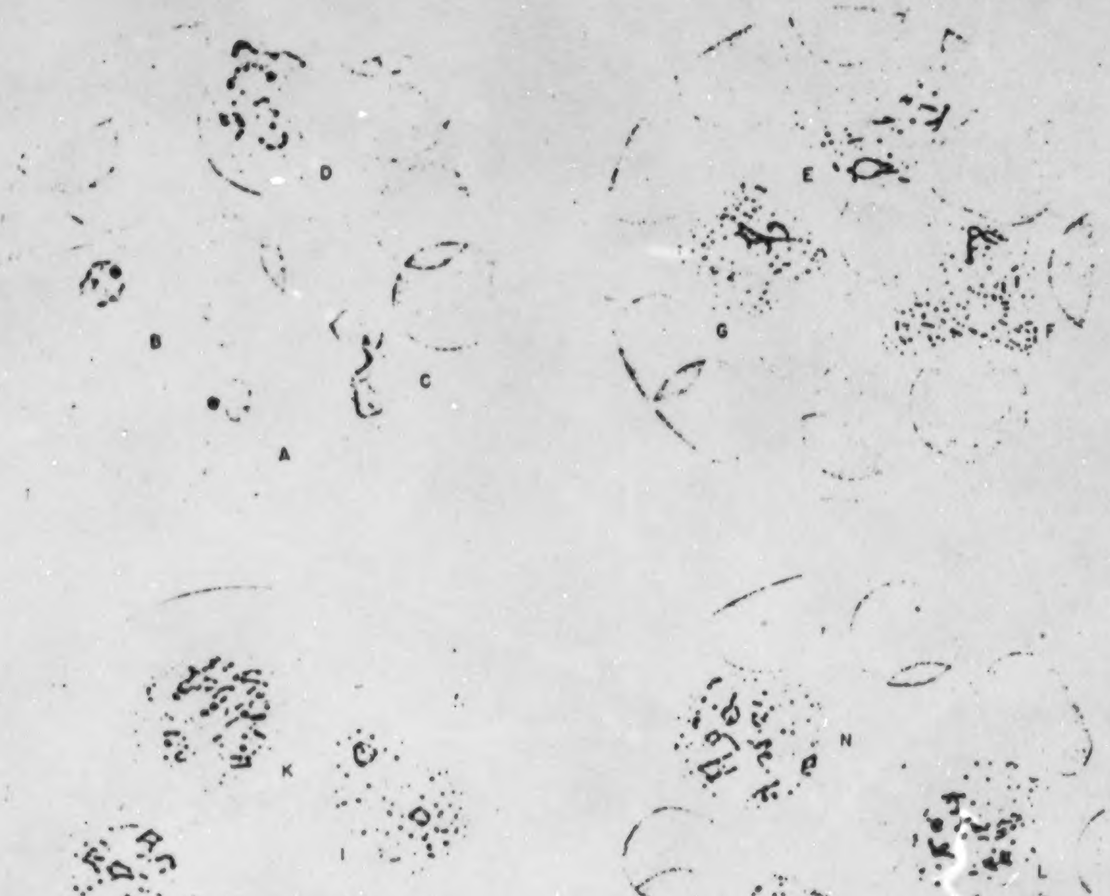
V. Mature Schizonts

O, P, and Q—Mature schizonts showing complete subdivision of nuclear chromatin and clumping of malarial pigment.



VI. Gametocytes or Sexual Forms

R—Male gametocyte or microgametocyte. Note diffuse nuclear chromatin.
S and T—Female gametocyte or macrogametocyte. Note compact chromatin.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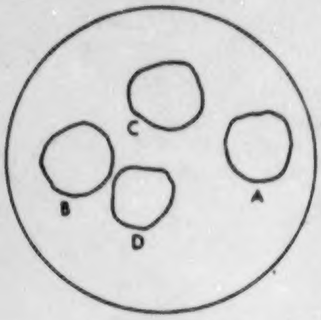
PLASMODIUM MALARIAE

(Quartan Malaria)

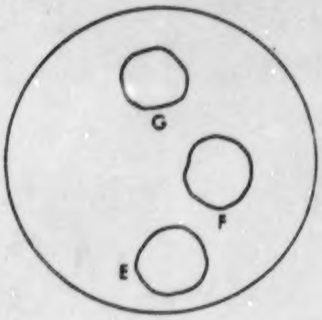
Color Plates

PLASMODIUM MALARIAE (Quartan Malaria) GIEMSA STAIN (x 2000)



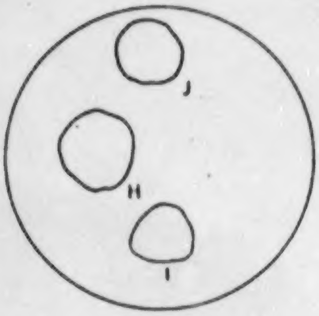
I. Young Trophozoite

A, B, and C—Progressively older ring forms.
D—Band trophozoite.



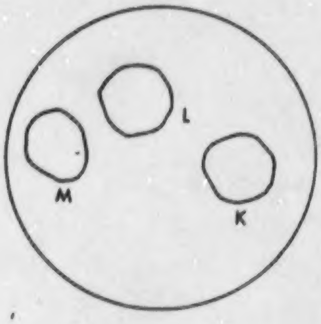
II. Half-grown Trophozoite

E—Ring form.
F and G—Mature trophozoites.
Note amount of pigment; compactness of cytoplasm.



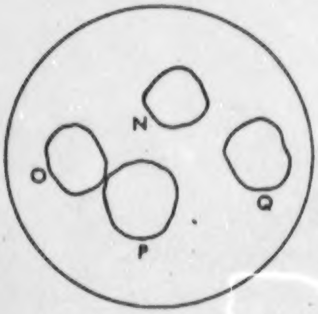
III. Young Schizonts

H—Band schizont.
I and J—Three- and five-nucleated schizonts.
Note large amount of pigment.



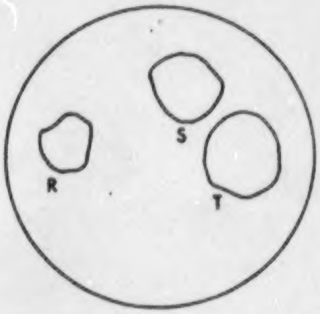
IV. Half-grown Schizonts

K, L, and M—Four- to six-nucleated schizonts. Note amount of pigment.



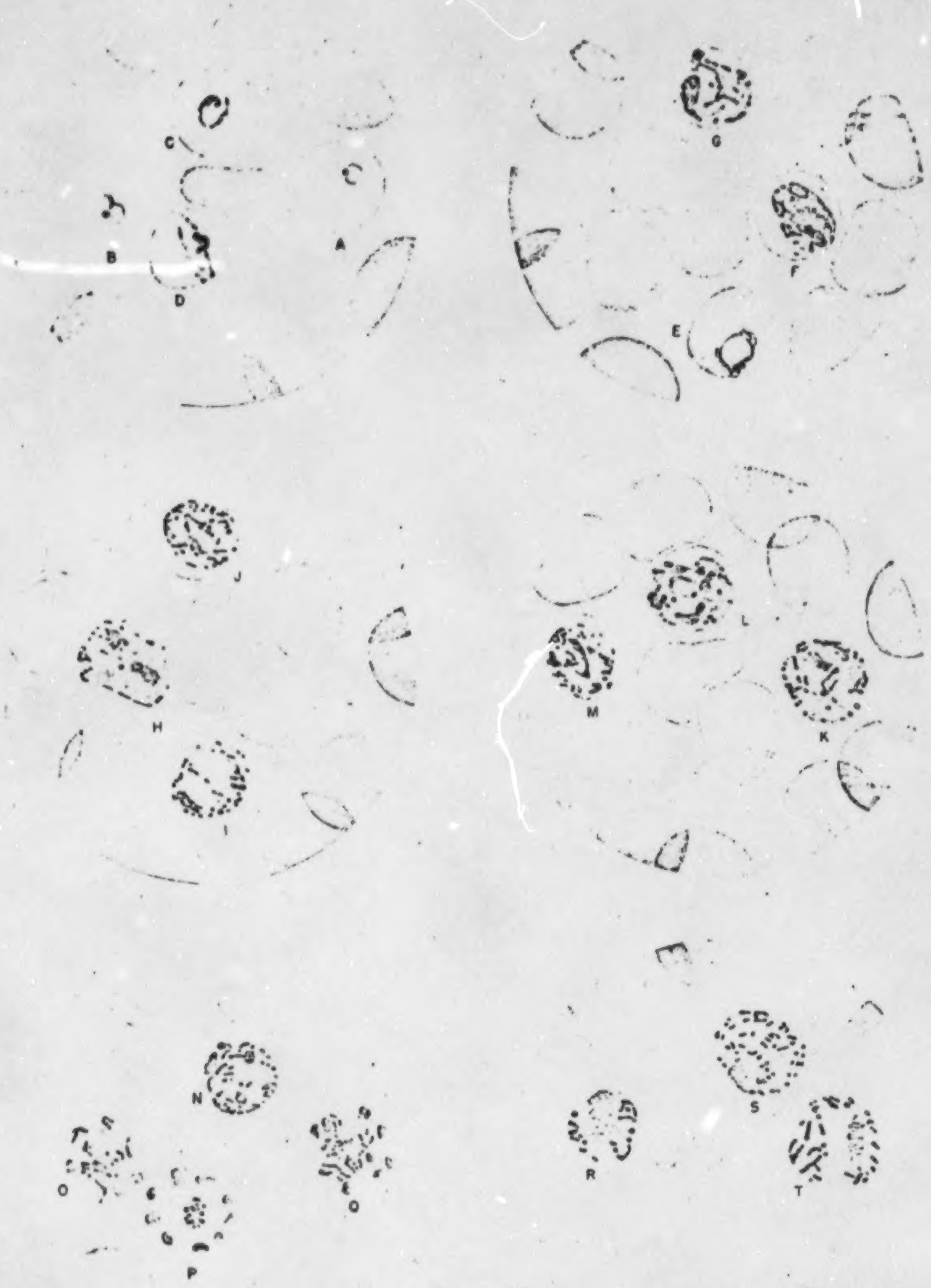
V. Mature Schizonts

N, O, P, and Q—Eight- or ten-nucleated schizonts ready to segment and release merozoites. Note "daisy" forms.



VI. Gametocytes or Sexual Forms

R and S—Male gametocytes or microgametocytes.
T—Female gametocyte or macrogametocyte.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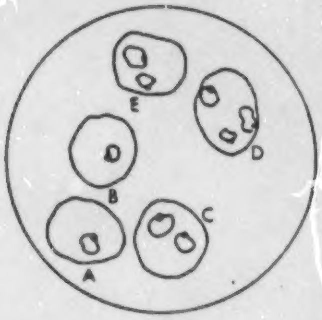
PLASMODIUM FALCIPARUM

(Estivo-Autumnal Malaria)

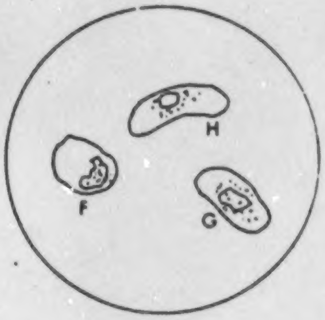
Color Plates

PLASMODIUM FALCIPARUM (Estivo-Autumnal Malaria) GIEMSA STAIN (x 2000)



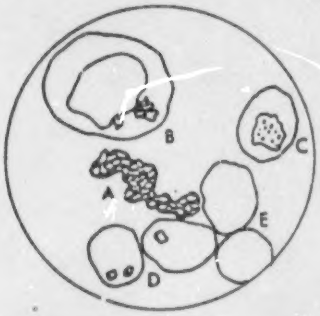
I. Trophozoites in Peripheral Blood

A—Young ring forms.
B, C, and D—Young trophozoites.
Note multiple infections of cells and appliqué forms.
E—These are oldest forms normally found in peripheral blood.



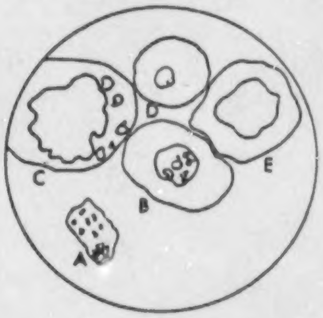
II. Gametocytes or Sexual Forms in Peripheral Blood

F—Gametocyte folded over.
G—Microgametocyte or male gametocyte. Note diffuse chromatin.
H—Female or macrogametocyte. Note compact chromatin.



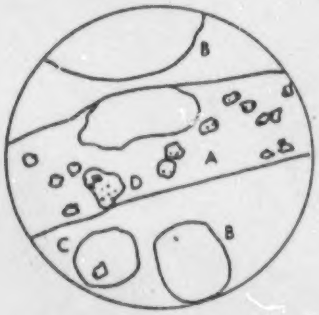
III. Impression Smear of Spleen

A—Free pigment of splenic pulp.
B—Pigment in macrophage.
C—Half-grown schizont in cell.
D—Parasitized red cells.
E—Lymphocytes.



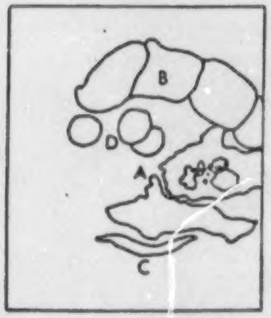
IV. Impression Smear of Bone Marrow

A—Free pigment granules.
B—Nucleated red cell.
C—Pigment in macrophage.
D—Parasitized red cell.
E—Eosinophile.



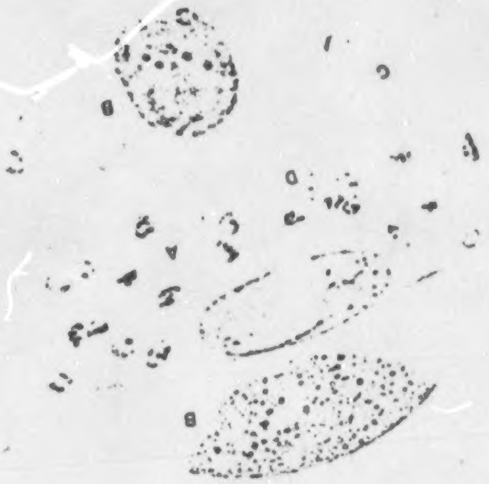
V. Impression Smear of Brain

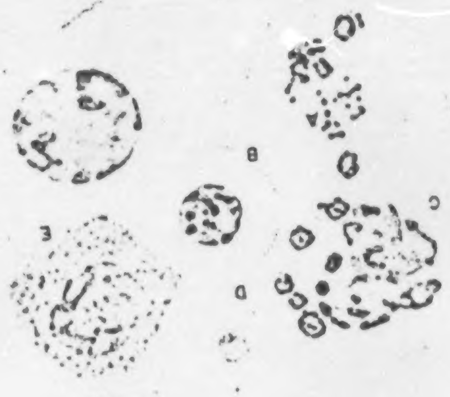
A—Capillary blocked with parasitized erythrocytes.
B—Glial cells.
C—Trophozoite in red cell.
D—Maturing schizont.

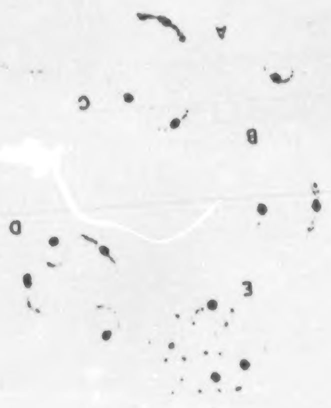


VI. Section of Liver

A—Küpffer cells with pigment.
B—Hepatic cells.
C—Endothelial cell.
D—Free red blood cells.







Chapter 5

LEISHMANIASIS AND TRYPANOSOMIASIS

Laboratory procedures for diagnosis of *Leishmania donovani* include culture of material taken from spleen, liver, bone marrow, lymph node or peripheral blood. Any of the above listed materials can also be stained with Giemsa's or Wright's stain and examined directly for Leishman-Donovan bodies and these materials can also be passed intraperitoneally into hamsters. To inoculate cultures, introduce aseptically amounts up to 1 ml of blood, bone marrow, or macerated tissue from spleen, lymph node, or liver by placing it in the fluid overlay. Incubate 3–14 days at 22° C.–25° C., checking daily after the third day for the characteristic leptomonad forms.

Laboratory procedures for diagnosis of infections with *Leishmania tropica* and *Leishmania brasiliensis* include staining and direct examination of exudate taken from the indurated margins of skin ulcers and the culture of such material on suitable media. Inoculum in amounts up to 1 ml is introduced in the fluid overlay and cultures are incubated at 22° C.–25° C. for 3–14 days with daily checks for characteristic leptomonad forms after the third day.

Diagnostic methods which may be employed for the detection of infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* vary with the stage of disease. In the earlier febrile stage the organisms are most likely to be recovered from the blood or macerated tissue from an enlarged lymph node. Methods that may be used are direct examination of stained or unstained smears, inoculation into rats or mice, and culture on suitable media. Cultivation of these two species is difficult, however. In the advanced stage of these diseases direct examination of spinal fluid and culture or animal inoculation of spinal fluid may be performed. In any of the various suggested laboratory procedures recovery of organisms may be facilitated by concentration techniques employing centrifugation. Ten ml quantities of blood or possibly lesser quantities of lymph gland juice are centrifuged at 900–1,000 rpm for 3 minutes. The supernate is pipetted off and centrifuged at 1,800–2,000 rpm for 20 minutes. Sediment can then be examined either stained or unstained and it can also be passed into experimental animals or culture. To concentrate trypanosomes in spinal fluid, centrifuge 5 ml of fluid as 1,500–2,000 rpm for 20 minutes. Again the sediment is examined either stained or unstained. The sediment can also be passed into culture or experimental animals.

The demonstration of *Trypanosoma cruzi* in the blood is difficult since the organisms occur in the peripheral blood only during the acute stage and during febrile periods in the chronic stage of the disease. Negative findings in direct examination of either fresh or stained blood films should be followed by inoculation of cultures. To inoculate cultures place ½–2 ml quantities of blood into the fluid overlay of suitable culture media. Animal inoculation should also be performed in mice, guinea pigs, or rats (free from *T. lewisi*). Inoculate experimental animals with ½–2 ml quantities of blood depending upon the size of the animal utilized. In both culture and experimental animals *T. cruzi* is much more readily recovered than the organisms of African sleeping sickness. Both cultures and animals should be checked periodically from the second through the fourth week following inoculation. Trypanosomes also revert to leptomonad forms in culture.

PREPARATION OF MEDIA FOR THE CULTURE OF TRYPANOSOMES AND LEISHMANIA

Diamond's SNB 9 Medium:

Materials and Equipment:

Water bath	
Test tubes	15.00 ml
Penicillin-Streptomycin solution	
Cotton, absorbent	
Neopeptone (Difco)	18.00 gm
Sodium chloride	4.95 gm
Distilled water	1000.00 ml
Agar	18.00 gm
Defibrinated rabbit's blood[1]	0.5–1.25 ml

Technique:

1. To prepare base stock add reagents in the order listed and dissolve thoroughly by supplying moderate heat if necessary by means of water bath. Tube medium in 5 ml quantities in 15 ml test tubes, plug tubes with cotton and autoclave for 20 minutes at 15 pounds pressure.
2. To prepare the medium melt it by bringing it up to a temperature of 45°–50° C. To each tube add ½ ml of defibrinated rabbit's blood, mix thoroughly, and cool in slants. The medium can be enriched by adding 1¼ ml defibrinated rabbit's blood to each tube for initial isolation of flagellates. The addition of 1,000 units of penicillin and 1,000 units of streptomycin to each tube is also recommended for initial isolation of flagellates.
3. To prepare overlay add neopeptone and sodium chloride to distilled water in quantities indicated in the base stock formula above and sterilize in the autoclave. When slants have hardened add ½ ml of overlay to each tube.

Kelser's Medium for the Cultivation of Trypanosoma Cruzi:

Materials and Equipment:

Flask, Erlenmeyer	1,000 ml
Water bath	
Cotton, absorbent	
Paper, litmus	
Flask, Erlenmeyer	250 ml
Pipette	10 ml
Test tubes	15 ml
Paraffin	
Bacto-Beef	25.0 gm
Bacto-Peptone	12.5 gm
Sodium chloride	3.5 gm
Bacto-Agar (granular)	5.0 gm
Dextrose solution, 1 percent	10.0 ml
Distilled water	500.0 ml
Sodium hydroxide solution[2]	1 N
Defibrinated rabbit or guinea pig blood[3]	10.0 ml

Technique:

1. To prepare base stock place 500 ml of distilled water in a 1,000 ml Erlenmeyer flask and add the Bacto-Beef.

Heat this mixture in a water bath at 55° C. for 1 hour. Following heating add the Bacto-Peptone and sodium chloride and bring the water bath to boiling. At this temperature heat for 5 minutes, shaking the flask several times to bring all ingredients into solution. Filter the broth through absorbent cotton 2 times. Using 1 N sodium hydroxide solution and litmus make the broth neutral. Following precise neutralization of the medium again place it in a hot water bath (boiling) and add the Bacto-Agar. When the Bacto-Agar is thoroughly dissolved dispense the medium in 100 ml amounts in Erlenmeyer flasks and sterilize in the autoclave at 12 pounds pressure for 30 minutes. The base stock can then be stored in the refrigerator until ready for use.

2. To prepare the medium for use place an Erlenmeyer flask containing 100 ml of the stock in a hot water bath

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[1] Quantity required for each tube of medium.

[2] To prepare 1 N sodium hydroxide, place 4 gm sodium hydroxide in a 100 ml graduate and q.s. to 100 ml with distilled water.

[3] Quantity required for each 100 ml of stock medium.

until the agar is melted. Lower the temperature of the medium to 50° C. and add aseptically the 10 ml of 1 percent dextrose solution and 10 ml of defibrinated blood. With a sterile 10 ml pipette transfer 5 ml quantities of the medium to 15 ml test tubes. Plug the tubes with sterile cotton, slant, and let stand until hardened. Paraffin the cotton plugs and place immediately in the refrigerator overnight to assure maximum water of condensation. To test for sterility incubate at 37° C. overnight. Store in refrigerator until ready for use.

NNN Medium for Leishmania:

Materials and Equipment:

Flask, Erlenmeyer	1,000 ml
Flasks, Erlenmeyer	250 ml
Paper, litmus	
Pipette	5 ml
Test tubes	15 ml
Bacto-Agar	14 gm
Sodium chloride	6 gm
Distilled water	900 ml
Sodium hydroxide solution [3]	1 N
Rabbit or guinea pig defibrinated blood [4]	10 ml

Technique:

1. To prepare base stock mix the distilled water, Bacto-Agar, and sodium chloride in an Erlenmeyer flask. Bring to a boil and then neutralize with 1 N sodium hydroxide using litmus paper as an indicator. Distribute the stock medium in suitable containers in 150 ml quantities. Sterilize at 12 pounds pressure for 15 minutes and store in refrigerator until ready for use. The stock medium will keep for several months at refrigeration temperature.
2. When ready to add the defibrinated blood heat a flask of base stock to boiling, then cool to 50° C. Add 10 ml of defibrinated blood aseptically, mix thoroughly and pipette 5 ml quantities of medium into 15 ml test tubes. Slant and when the medium has hardened place overnight in the refrigerator to enhance formation of water of condensation. Incubate at 37° C. overnight to test for sterility before use.

Offutt's Medium for Cultivation of T. Cruzi, T. Lewisi, and the Leishmanias:

Materials and Equipment:

Flask, Erlenmeyer	1,000 ml
Water bath	
Flasks, Erlenmeyer	250 ml
Test tubes	15 ml
Cotton, absorbent	
Paraffin	
Bacto Blood-Agar Base	40 gm
Distilled water	1,000 ml
Defibrinated rabbit's blood [5]	10–20 ml
Sterile buffered saline or sterile Locke's solution [6]	0.5–1 ml

Technique:

1. To prepare base stock add the Bacto Blood-Agar Base to the distilled water in an Erlenmeyer flask, soak for 5 minutes, mix thoroughly and heat in a water bath until the agar is thoroughly dissolved. Distribute base stock in 200 ml amounts, and sterilize in the autoclave at 15 pounds pressure for 20 minutes. The stock is stored in the refrigerator until ready for use.
2. When ready to add the defibrinated blood heat one flask of the base stock to boiling, then cool to 45°–50° C. Add asceptically 10–20 ml of defibrinated rabbit's blood, mix thoroughly and tube in 4–5 ml amounts in sterile 15 ml test tubes. Allow the medium to solidify in slants. A fairly short slant with deep butt is preferred. Add ½–1 ml

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[3] To prepare 1 N sodium hydroxide, place 4 gm sodium hydroxide in a 100 ml graduate and q. s. to 100 ml with distilled water.

[4] Quantity required for each 150 ml of stock medium.

[5] Quantity required for each 200 ml of stock medium.

[6] Quantity required for each tube of medium.

of sterile Locke's solution or sterile buffered saline to each tube. Test for sterility by incubating for 24 hours at 37° C. Paraffin cotton plugs to prevent evaporation of overlay.

Senekjie's Modified Medium for Trypanosoma Cruzi and Leishmania:

Materials and Equipment:

Flask, Erlenmeyer	1,000 ml
Water bath	
Funnel	
Paper, filter	
Flasks, Erlenmeyer	250 ml
Test tubes	15 ml
Pipette	10 ml
Cotton, absorbent	
Paraffin	
Bacto-Beef	50 gm
Distilled water	1,000 ml
Neopeptone	20 gm
Sodium chloride	5 gm
Noble special agar [7]	20 gm
Defibrinated rabbit's blood [8]	10 ml
Sterile Locke's solution [9]	2 ml

Technique:

1. To prepare base stock dissolve the Bacto-Beef in the distilled water and heat to 50° C. for 1 hour in a hot water bath. Raise the temperature to 80° C. for 5 minutes to coagulate proteins, then filter through filter paper. Add the neopeptone, sodium chloride and agar and adjust pH to 7.2–7.4. Autoclave at 15 pounds pressure for 20 minutes. Distribute base stock in Erlenmeyer flasks in 90 ml quantities and store in the refrigerator.
2. When ready to add the defibrinated blood place an Erlenmeyer flask containing 90 ml of the base stock in a hot water bath and heat until agar is melted. Lower the temperature to 50° C. and add aseptically 10 ml of defibrinated rabbit's blood. With a sterile 10 ml pipette transfer 5 ml quantities of the medium to 15 ml test tubes. Plug the tubes with sterile cotton, slant, and let stand until hardened. Overlay with 2 ml of sterile Locke's solution. Paraffin the cotton plugs and place in incubator overnight at 37° C. to test for sterility. Store in refrigerator until ready for use.

Table 1. A Comparative Table of the Medically Important Trypanosomes

Characteristics	*Trypanosoma gambiense*	*Trypanosoma rhodesiense*	*Trypanosoma cruzi*
Common name of disease produced.	African sleeping sickness or more specifically Gambian sleeping sickness.	African sleeping sickness or more specifically Rhodesian sleeping sickness.	Chagas' disease.
Geographical distribution.	Wide area in tropical West Africa from Senegal to Loanda extending inland along the large rivers, particularly the Congo and Niger. Newer areas include Southern Sudan, Uganda, and the Great Lakes region.	Eastern Africa from Kenya to southern Rhodesia and northern Mozambique, and inland across Tanganyika to Uganda and eastern Congo.	Extends from pampas of Argentina to deserts and canyons of southern California, Arizona, and Texas. Few cases occur in Mexico and Central America, common in many parts of Venezuela, French Guiana, Brazil, Bolivia, Chile, Paraguay, and Uruguay.

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[7] Noble special agar is a highly purified agar available under corresponding trade name.

[8] Quantity required for each 90 ml of stock medium.

[9] Quantity required for each tube of medium.

Table 1. A Comparative Table of the Medically Important Trypanosomes—Continued

Characteristics	*Trypanosoma gambiense*	*Trypanosoma rhodesiense*	*Trypanosoma cruzi*
Vector and mode of transmission.	Flies of the genus *Glossina*. "Metacyclic" infective typanosomes occur in the salivary glands. Transmitted by bite. Mechanical transmission is possible up to 30 minutes after fly feeds on infected individual.	Flies of the genus *Glossina*. "Metacyclic" trypanosomes occur in the salivary glands. Transmitted by bite. Mechanical transmission possible as with *T. gambiense*.	Bugs of the genus *Triatoma*. "Metacyclic" infective forms occur in the anal region. Transmitted through contamination of broken skin by insect droppings.
Localization of the parasites within the vertebrate host.	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system. Invasion of the central nervous system takes place later in the course of the disease than in infections with *T. rhodesiense*.	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system. This is usual course of the disease in the case of infections with *T. rhodesiensc*.	Organism invades cells of the heart, voluntary muscles, various glands, and late in the course of the disease, the central nervous system. Organisms usually scarce in the circulating blood.
Clinical symptoms of the disease.	Course irregular, generally milder than Rhodesian type of the disease and of much longer duration. Initial symptoms include fever and headache recurring irregularly followed by weakness, enlarged glands, edema and muscular tenderness. In the late stages there is physical depression, stupor, unconsciousness, coma, and death.	Organism is apparently more virulent and death frequently occurs within 3 or 4 months following infection. Initial symptoms include fever and headache recurring irregularly followed by weakness, enlarged glands, edema, and muscular tenderness. In the late stages there is physical depression, stupor, unconsciousness, coma and death.	Initial stage swelling of eyelids, and conjunctiva, and edema of other parts of face, usually one side only. Lymph glands of neck swell; there may be severe headache, and prostration with more or less continuous fever. In the chronic stage there is extensive hard edema, inflamed glands and enlargement of liver and spleen. Late stage is characterized by extreme weakness, convulsions, paralysis and disturbance of heart function. Intercurrent diseases common and frequently immediate cause of death.
Laboratory diagnosis.	Fresh specimen, thin- or thick-stained smear, hanging drop, centrifugalization, xenodiagnosis, animal inoculation, culture, spinal fluid examination, or biopsy of of lymphatics.	Same as for *T. gambiense*, the method of choice usually depending upon the stage of the disease.	Same as for *T. gambiense*, the method of choice generally depending upon the stage of the disease.

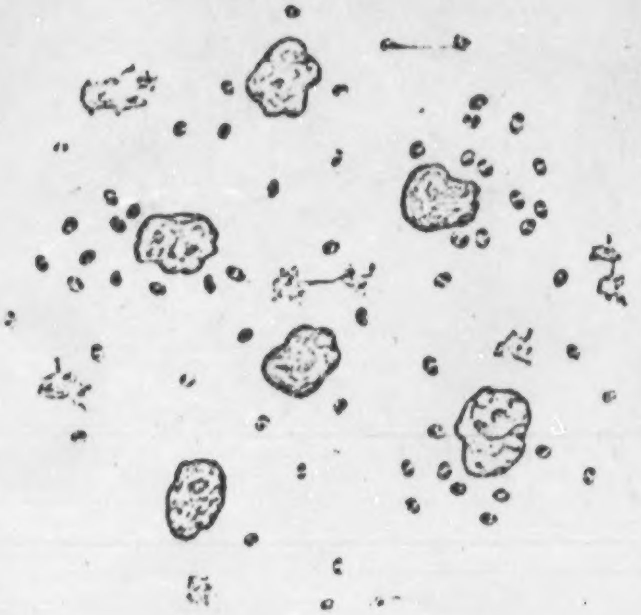
Table 2. A Comparative Table of the Medically Important Leishmania

GENERAL CHARACTERISTICS OF THE GENUS *LEISHMANIA:* 1. Rounded forms containing a nucleus and kinetoplast but lacking a flagellum. 2. Any of the other three types of "hemoflagellates" (Leptomonas, Crithidia, and Trypanosoma) may assume this form and may also be developed out of it. 3. In addition to the Trypanosoma this is the only other form of "hemoflagellate" which may be found in the circulating blood of man. 4. The three presently recognized types of human Leishmaniasis are all caused by morphologically indistinguishable parasites, separable on basis of clinical manifestations and geographical distribution. 5. Dogs constitute the principal reservoir animals of Kala-azar and Oriental sore.

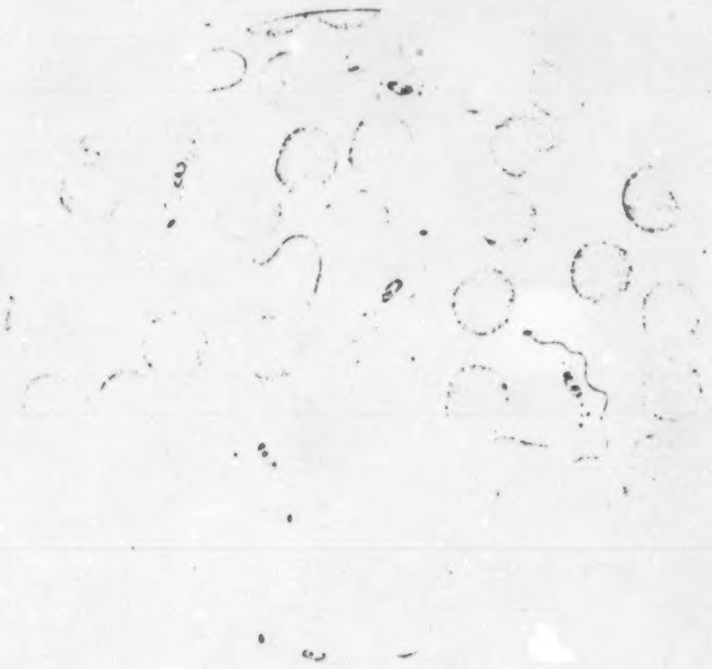
Characteristics	*Leishmania donovani*	*Leishmania tropica*	*Leishmania braziliensis*
Common name of disease produced.	Kala-azar, or visceral leishmaniasis.	Oriental sore, or cutaneous leishmaniasis.	Espundia, or South American mucocutaneous leishmaniasis.
Geographical distribution.	In the old world it is common in India, North China, and the Sudan. Around the Mediterranean and in western and middle Asia infantile type of disease occurs. In recent years it has been discovered that the disease is widespread in South America (parts of Brazil, Argentina, Paraguay, Bolivia, and Venezuela).	Common in tropical cities of eastern Mediterranean, usually in children under 3 years. In these regions dogs are frequently infected and are an important reservoir of the disease.	Form of cutaneous leishmaniasis which is frequently followed by involvement of mucous membranes of nose and pharnyx. Occurs over large area of tropical America from Yucatan in Mexico through Central America into northern Argentina.
Vector and mode of transmission.	Flies of the genus *Phlebotomus* are proven natural vectors although other insects may transmit the disease. Transfer of fomites from infected human or reservoir animals can transmit the disease. Transmission generally by bite of the fly.	Generally by flies of the genus *Phlebotomus* although direct mechanical transmission is definitely possible. Most common vector is *Phlebotomus papatasi*, a sandfly found wherever this disease occurs. Transmission generally by bite of fly.	Highly probable that *Phlebotomus* flies are generally responsible. This cycle has not been thoroughly worked out as has that of the other two forms, but naturally infected *Phlebotomus* have been found in areas where the disease is endemic.
Localization of the parasites.	Leishman-Donovan bodies are widely distributed within the body but special habitat seems to be large endothelial cells of blood vessels and lymphatics. Abundant in spleen, liver, bone marrow, and sometimes found in circulating blood within the monocytes.	Parasites are found in the dermal tissues of infected sores where increased numbers of large monocytes and other reticulo-endothelial cells are literally packed with them.	Initially parasites are localized in cutaneous sores as in the case of *L. tropica*. As disease advances they localize in mucous membranes of nose and pharnyx.

Table 2. A Comparative Table of the Medically Important Leishmania—Continued

Characteristics	*Leishmania donovani*	*Leishmania tropica*	*Leishmania braziliensis*
Common name of disease produced.	Kala-azar, or visceral leishmaniasis	Oriental sore, or cutaneous leishmaniasis.	Espundia, or South American mucocutaneous leishmaniasis.
Clinical symptoms of the disease.	Onset is accompanied by irregular fever, rheumatic aches, anemia, and progressive emaciation. Macrophages increase tremendously in number and size so that involved organs including liver and spleen increase markedly in size also. Leukocytes decrease in number, general edema of skin is present. Untreated persons die within a few weeks to several years.	Begins as small red papule which enlarges to an inch or more in diameter. In later stages these develop into unsightly ulcerating sores. Sores persist for a few months to a year or more. They finally heal spontaneously and generalized invasion of the body does not occur.	Typical cases begin initially in a manner similar to Oriental sore except that there is greater tendency for the sores to spread over extensive areas and for numerous sores to appear. In later stages of the disease secondary ulcerations occur in nasal cavities, mouth and pharynx, these generally developing several months to several years after cutaneous lesions appear. Mucocutaneous involvement results in clogging of nostrils, fetid breath, affection of organs of smell, hearing and sometimes sight. In advanced stages the nasal septum and surfaces of the palate are destroyed. Death always occurs in advanced untreated cases, but patients may live several years.
Laboratory diagnosis	Liver or spleen puncture, biopsy of lymph glands, with culture of material recovered, culture of blood, and direct examination of stained blood smears.	Animal inoculation, culture and direct examination of exudates from sores (latter procedure generally confirms positive cases).	Intradermal tests are useful. Direct examinations of exudates from involved areas, culture and animal inoculation are practical laboratory procedures.



Leishmaniasis in macerated hamster spleen. Note intracellular and extracellular Leishman-Donovan bodies.



Trypanosoma lewisi in blood smear from an infected rat.

Table 1. Myiasis in Man

Specific Myiasis

COMMON NAME OF SPECIES	GROUP OR SPECIES NAME	HOST RELATIONSHIP	REMARKS	KNOWN GEOGRAPHICAL DISTRIBUTION
Congo floor maggots.	*Auchmeromyia luteola.*	Sucks blood at night only.	Larvae hide in ground during daytime.	Tropical Africa.
Tumbu flies	*Cordylobia anthropophaga*	Young larvae invade unbroken skin.	Larvae normally leave body after 8–9 days.	Tropical Africa.
Human botflies	*Dermatobia hominis.*	Larvae invade exposed skin areas.	Fly glues eggs to mosquito or other "carrier" insect.	Tropical South America, Central America, and Mexico.
Horse botflies	*Gasterophilus* spp	Larvae wander beneath skin.	Three species are normal parasites in gut of horse.	World-wide.
Cattle botflies	*Hypoderma* spp	Larvae wander beneath skin.	Normal parasites beneath skin on backs of cattle.	World-wide.
Sheep botflies	*Oestrus ovis*	Larvae invade nose, pharynx, conjunctival sac.	Normal parasites in naso-pharnyx of sheep.	World-wide.
Head botflies of horses.	*Rhinoestrus purpureus.*	Larvae invade nasopharynx and conjunctival sac.	Normal parasites in head passages of horses.	Southern and Eastern Europe, N. Africa, Asia Minor.
Primary screw-worm flies.	*Callitroga americana.*	Larvae invade nose, ears, sinuses, and wounds (rarely unbroken skin).	Mortality, 8 percent	Warmer regions of the Western Hemisphere.
Old World screw-worm flies.	*Chrysomyia bezziana.*	Larvae invade nostrils, ears, conjunctiva, vagina, or open wounds.	Larvae complete development and drop out in 7–14 days.	Oriental and Ethiopian Regions.
Flesh flies	*Wohlfahrtia magnifica.*	Invade nose, ears, wounds of all types.	May cause death	Mediterranean, Near East, and Russia.
	W. vigil	Invade unbroken skin.	Parasitize babies, especially in neck region.	Nearctic Region.
	W. meigenii	Invade unbroken skin.	Parasitize babies, especially in neck region.	Western U.S.A.
Semi-Specific Myiasis				
Common screw-worm flies.	*Callitroga macellaria.*	Invade open wounds	Normal in decaying flesh.	Widespread in Western Hemisphere.
Green bottle flies.	*Lucilia* spp	Usually attack only diseased tissue.	Formerly used in surgery and treatment of osteomyelitis	World-wide.

Table 1. Myiasis in Man—Continued

COMMON NAME OF SPECIES	GROUP OR SPECIES NAME	HOST RELATIONSHIP	REMARKS	KNOWN GEOGRAPHICAL DISTRIBUTION
Blue bottle flies.	*Calliphora* spp. . . . *Cynomyia* spp.	Invade wounds, cutaneous ulcers, and malodorous apertures.	Usually attack only diseased tissue.	World-wide.
Black bottle flies.	*Phormia* spp. . . .	Invade wounds, cutaneous ulcers, and malodorous apertures.	Formerly used in treatment of osteomyelitis.	World-wide.
Flesh flies	*Sarcophaga* spp. . . .	Invade wounds, cutaneous ulcers, and malodorous apertures; may penetrate unbroken skin.	Many species are viviparous.	World-wide.
Stable flies	*Stomoxys calcitrans*.	Invades open wounds. .	Rather rare in man. . . .	World-wide.
Houseflies.	*Musca* spp.	Invade open wounds. .	Presumed to be a secondary invader.	World-wide.
		Accidental Myiasis—Intestinal		
Houseflies. . . . . . Green bottle flies. Blue bottle flies. Flesh flies. . . . . . Latrine flies . . . . Cheese skippers. Rat tail maggots	*Musca domestica* *Lucilia* spp. *Colliphora* spp. Family Sarcophagidae. *Fannia scalaris*. *Piophila casei*. *Tubifera* spp.	Inhabit various portions of the intestinal tract.	Infection acquired by ingestion of eggs or larvae or by flies depositing eggs or larvae on anus during defecation.	More or less generally distributed throughout the world.
		Accidental Myiasis—Genito-Urinary		
Houseflies. . . . . . Cheese skippers. Rat tail maggots Latrine flies. . . . Green bottle flies Blue bottle flies. Flesh flies.	*Musca domestica*. . . *Piophila casei*. *Tubifera* spp. *Fannia* spp. *Lucilia* spp. *Calliphora* spp. *Sarcophaga* spp.	Urinary tract, including bladder; genital passages of females.	Infection acquired by migration of larvae from intestinal tract or by flies depositing eggs or larvae on genital aperture, especially of females.	Not common but occurs widely the world over.

Table 2. Summary of the More Important Arthropod Vectors of Human Disease

Exclusive of Myiasis

VECTOR	DISEASE	SPECIFIC AGENT
Millipedes	Hymenolepiasis	*Hymenolepis diminuta.*
Copepoda (water fleas)	Diphyllobothriasis	*Diphyllobothrium latum.*
	Dracontiasis	*Dracunculus medinensis.*
	Gnathostomiasis	*Gnathostoma spinigerum.*
	Sparganosis	*Diphyllobothrium* spp.
Decapoda (crayfish, crabs, etc.).	Paragonimiasis	*Paragonimus westermani.*
Mites	Bertelliasis	*Bertiella studeri.*
	Richettsialpox	*Rickettsia akari.*
	Tsutsugamushi	*Rickettsia tsutsugamushi.*
Ticks	Boutonneuse fever	*Rickettsia conora.*
	Bullis fever	*Rickettsia* sp.
	Colorado tick fever	Filtrable virus.
	Encephalitis, Russian spring-summer	*Erro sylvestris virus.*
	Louping ill	Virus sp.
	Maculatum disease	*Rickettsia* sp.
	Plague	*Pasteurella pestis.*
	Q Fever	*Coxiella burneti.*
	Relapsing fever (endemic)	*Borrelia duttoni.*
	Spotted fever	*Rickettsia rickettsia.*
	Tularemia	*Pasteurella tularensis.*
Black flies	Onchocerciasis	*Onchocerca volvulus.*
	Tularemia	*Pasteurella tularensis.*
Cockroaches	Amebiasis	*Endamoeba histolytica.*
	Balantidiasis	*Balantidium coli.*
	Cholera	*Vibrio comma* (cholera).
	Dysentery, bacillary	*Shigella* spp.
	Food poisoning	*Staphylococcus* sp., *Salmonella* sp., *Streptococcus* sp.
	Giardiasis	*Giardia lamblia.*
	Gongylonemiasis	*Gongylonema pulchrum.*
	Hymenolepiasis	*Hymenolepis diminuta.*
	Poliomyelitis	Virus types 1, 2, 3.
	Paratyphoid fever	*Salmonella* spp.
	Typhoid fever	*Salmonella typhosa.*
Cone-nose bugs	Trypanosomiasis (Chagas)	*Trypanosoma cruzi.*
Deer flies	Loaiasis	*Loa loa.*
	Tularemia	*Pasteurella tularensis.*
Eye gnats	Conjunctivitis	Bacteria spp.
	Tropical ulcer	Mixed bacteria and spirochete flora.
	Trachoma	Virus.
	Yaws	*Treponema pertenue.*

Table 2. Summary of the More Important Arthropod Vectors of Human Disease—Continued

VECTOR	DISEASE	SPECIFIC AGENT
Fleas	Dipylidiasis	*Dipylidium caninum.*
	Hymenolepiasis	*Hymenolepis diminuta.*
	Plague	*Pasteurella pestis.*
	Typhus fever (endemic)	*Rickettsia typhi.*
Housefly and other filth flies	Amebiasis	*Endamoeba histolytica.*
	Bacillary Dysentery	*Shigella* spp.
	Balantidiasis	*Balantidium coli.*
	Cholera	Vibrio comma (cholera).
	Cysticercosis	*Taenia solium.*
	Food poisoning	*Staphylococcus* sp. *Salmonella* sp., *Streptococcus* sp
	Giardiasis	*Giardia lamblia.*
	Paratyphoid fever	*Salmonella* sp.
	Poliomyelitis	Virus types 1, 2, 3.
	Trachoma	Virus.
	Trichuriasis	*Trichuris trichiura.*
	Tropical ulcer	Mixed bacteria and spirochete flora.
	Typhoid fever	*Salmonella typhi.*
	Yaws	*Treponema pertenue.*
Lice	Relapsing fever (epidemic)	*Borrelia recurrentis.*
	Trench fever	*Rickettsia quintana.*
	Typhus fever (epidemic)	*Rickettsia prowazeki.*
Mosquitoes	Dengue	*Charon* sp. virus.
	Encephalitis (California; Equine, Eastern, Venezuelan, Western; Japanese B; Russian, spring-summer; West Nile).	Virus.
	Filariasis, Bancroft's	*Wuchereria bancrofti.*
	Filariasis, Malayan	*W. malayi.*
	Malaria	*Plasmodium* spp.
	Rift Valley fever	Virus.
	Tularemia	*Pasteurella tularensis.*
	Yellow fever	*Charon evagatus.*
Moths and beetles	Hymenolepiasis	*Hymenolepis diminuta.*
Biting gnats	Acanthocheilonemiasis	*Acanthocheilonema perstans.*
	Mansonelliasis	*Mansonella ozzardi.*
	Microfilaria streptocerca	*Acanthocheilonema streptocerca.*
Sand flies	Bartonellosis	*Bartonella bacilliformis.*
	Espundia	*Leishmania brasiliensis.*
	Kala-azar	*L. donovani.*
	Oriental sore	*L. tropica.*
	Sandfly fever	*Pappataci* virus.
Tsetse flies	Trypanosomiasis	*Trypanosoma gambiense, T. rhodesiense.*

Table 3. Summary of the More Important Human Diseases Transmitted by Arthropods

Bacterial Diseases

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Cholera	Nonbiting flies and cockroaches.	*Musca* spp. and domestic cockroaches.	World-wide
Conjunctivitis	Nonbiting flies	*Hemophilus* spp	United States.
		Hippelates collusor	California.
		H. flavipes	Coastal U.S.A. in Gulf region.
		Note: Undoubtedly other nonbiting Diptera the world over.	
Dysentery	Nonbiting flies and other arthropods living in close contact with humans.	Examples: *Musca* spp. and members of the family Blattidae.	World-wide.
Plague	Fleas	*Citellophilus tesquorum*	Mongolia.
		Chiastopsyllus rossi	South Africa.
		Diamanus montanus	California and Oregon.
		Dinopsyllus lypusus	South Africa.
		Hoplopsyllus anomalus	California and Oregon.
		Nosopsyllus fasciatus	Temperate zone.
		Oropsylla silantiewi	Mongolia.
		Pulex irritans	World-wide.
		Rhopalopsyllus cavicola	Argentina and Ecuador.
		Synosternus pallidus	West Africa.
		Xenopsylla astia	India, Ceylon, Burma, Mesopotamia, Mombasa.
		X. brasiliensis	Belgian Congo, Kenya, Uganda, Bechuanaland.
		X. cheopis	World-wide.
		X. eridos	South Africa.
		X. nubicus	Tropical East and West Africa.
Tularemia	Deer flies, ticks, and mosquitoes.	*Amblyoma americanum*	United States.
		Dermacentor andersoni	United States.
		D. variabilis	United States
		D. silvarum	U.S.S.R.
		Aedes cinereus	Sweden.
		Culex apicalis	U.S.S.R.
		Chrysops discalis	Western U.S.A.
Typhoid and Paratyphoid Fever	Flies and cockroaches	*Musca* spp. and other filth flies and cockroaches.	World-wide.
Helminths			
Bertielliasis	Mites	*Orbatid* mites	Africa, Asia, Mauritius, West Indies

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Cysticercosis (bladder worm).	Nonbiting flies..........	*Musca domestica* and other filth flies.	World-wide.
Diphyllobothriasis (fish tapeworm).	Copepods containing infective stage.	*Cyclops* spp.............. *Diaptomus* spp.	Europe and North America, Japan, and scattered areas of Africa.
Dipylidiasis (dog tapeworm).	Fleas or lice containing infective stage.	*Ctenocephalides canis C. felis* *Pulex irritans* *Trichodectes canis*	World-wide
Hymenolepiasis diminuta (rat tapeworm disease).	Arthropods containing infective stage.	Coleoptera................ Dermaptera Diplopoda Lepidoptera Orthoptera Siphonaptera	India, Russia, Japan, Italy, U.S.A.
Hymenolepiasis nana (dwarf tapeworm).	Beetles and fleas containing infective stage.	Siphonaptera............. Coleoptera	World-wide.
Trichuriasis (whipworm).	Nonbiting flies..........	*Musca* supp. and other filth flies.	World-wide.
Acanthocheilonemiasis....	Biting gnats..............	*Culicoides austeni C. grahami.*	Rain forests of West and Central Africa, tropical South America and New Guinea, and coastally in Trinidad and Panama.
Bancroft's filariasis......	Mosquitoes............	*Culex quinquefasciatus*......	World-wide.
		Culex pipiens............	Central China, Japan, and Egypt.
		Aedes aegypti............	West Africa, Belgian Congo, New South Wales, Surinam, West Indies.
		Anopheles albimanus.......	Caribbean area.
		Anopheles darlingi.........	Brazil.
		A. gambiae...............	West Africa, Belgian Congo, Zanzibar.
		A. punctulatus...........	New Guinea, various South Pacific islands.
		Aedes polynesiensis......... Note: Also 60 additional species of mosquitoes.	Numerous Polynesian islands.
Dracontiasis (guinea worm).	Copepods...............	*Cyclops* supp..............	Africa, India, Russia, Middle East.
Gongylonemiasis.	Cockroaches............	Blattidae.	Italy, Bulgaria, U.S., Russia, Ceylon, China.
Loaiasis (eye worm)......	Deer flies..............	*Chrysops dimidiata, C. distinctpennis, C. silacea.*	Tropical Africa, especially the Congo River basin.

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Malayan filariasis	Mosquitoes	*Anopheles barbirostris*	Indonesia, Malaya, and China.
		A. hyrcanus complex	Indonesia, Malaya, and China.
		A. umbrosus	Malaya.
		Mansonia annulata	Indonesia, Malaya, and Philippines.
		M. annulifera	Indonesia, Malaya, India, and Ceylon.
		M. Longipalpis	Malaya.
		M. indiana	Malaya.
		M. uniformis	Malaya.
Mansonelliasis (Ozzard's filariasis).	Biting gnats	*Culicoides furens*	West Indies, Central and South America.
Onchocerciasis (blinding filariasis).	Black flies	*Simulium callidum*	Guatemala, Southern Mexico.
		S. metallicum	Guatemala, Southern Mexico.
		S. ochraceum	Guatemala, Southern Mexico.
		S. damnosum	Tropical Africa.
		S. neavei	Tropical Africa.
Paragonimiasis (lung flukes).	Infective stage in crabs and crayfish.	*Astacus japonicus*	Orient.
		A. similis	Orient.
		Eliocheir sinensis	China.
		Potoman dehaani	Africa, China, South America.
		P. denticulatus	Orient.
		Pseudothlphusa iturbei	Venezuela.
Protozoa			
Amebiasis	Nonbiting flies	*Musca* spp. and other filth flies and cockroaches.	World-wide.
Balantidiasis	Nonbiting flies	*Musca* spp. and other filth flies.	World-wide.
Giardiasis	Nonbiting flies	*Musca* spp. and other filth flies.	World-wide.
Espundia (muco-cutaneous leishmaniasis).	Sandflies	*Phlebotomus* spp	Central and South America.
Kala-azar (visceral leishmaniasis).	Sandflies	*Phlebotomus argentipes*	India.
		P. chinensis	China.
		P. intermedius	South America.
		P. longipalpis	Brazil.
		P. major	Greece.
		P. perniciosis	Italy, Sicily.
		P. sergenti mongolensis	China.

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Oriental sore (cutaneous leishmaniasis).	Sandflies	*Phlebotomus papatasii*	India.
		Phlebotomus spp	Africa, Asia.
Malaria	Mosquitoes	Numerous sp. of mosquitoes of the genus *Anopheles*.	64° North to 32° South in tropical and temperate zones except certain islands.
African Trypanosomiasis.	Testse flies	*Glossina palpalis*	Equatorial Africa.
		G. tachinoids	West Africa.
		G. morsitans	East Africa.
		G. swynnertoni	Tanganyika.
South American Trypanosomiasis.	Assassin bugs	*Panstrongylus* spp	Panama, South America.
		Rhodnius spp	South America, Mexico.
		Triatoma spp Note: Also other genera and species	U.S.A., South America, Mexico, and Central America.
		Rickettsia	
Typhus, endemic	Fleas	*Ctenocephalides felis*	Texas.
		Nosopsyllus fasciatus	Europe, North America.
		Xenopsylla cheopis	World-wide.
		X. astia	Orient.
Typhus, epidemic	Lice	*Pediculus humanus*	World-wide.
Trench fever	Lice	*Pediculus humanus*	Western Ukraine.
Rickettsialpox	Mites	*Allodermanyssus sanguineus*.	North Atlantic Seaboard, U.S.A.
Typhus, scrub (Tsutsugamushi).	Mites	*Trombicula akamushi, T. deliensis,* and *T. fugi.*	Japan, Australasian region, East Indies, and the Philippines.
Boutonneuse fever	Ticks	*Ambyloma hebraeum, Haemaphysalis leachi, Hyalomma rufipes, Rhipicephalus appendiculatus, R. simus.*	East, Central and South America.
		Rhipicephalus sanguineus	Mediterranean Basin, the Crimea and India.
		Ixodes holocyclus	North and South Queensland.
		Dermacentor nuttali, D. silvarum and *Haemaphysalis concinna.*	Russia and Eastern and Central Siberia.
Bullus fever (Lone Star fever).	Ticks	*Ambyloma americanum*	Texas.

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Spotted fever	Ticks	*Ambyloma americanum*	Oklahoma and Texas.
		Dermacentor andersoni	Western United States.
		D. variabilis	Eastern and Southern United States.
		Ambyloma brasilensis, A. cajennense, A. striatum.	Panama, Colombia, and Brazil.
		Rhipicephalus sanguineus	Mexico.
Maculatum disease	Ticks	*Ambyloma maculatum*	South Central and Southeastern United States.
Q fever	Ticks	Seventeen tick species have thus far been found naturally infected.	World-wide.
Central Nervous System Viral Infections			
California virus	Mosquitoes	*Aedes dorsalis* and *Culex tarsalis.*	San Joaquin Valley, California.
Eastern equine encephalitis.	Mosquitoes, mites, and lice.	*Culiseta melanura*	Louisiana, U.S.A.
		Mansonia perturbans	Georgia, U.S.A.
		Dermanyssus gallinae and *Eomenacanthus stramineus.*	Tennessee, U.S.A.
Western equine encephalomyelitis.	Mosquitoes and mites	*Culex tarsalis, Culex* spp., *Aedes dorsalis, Culiseta inornata, Anopheles freeborni, Dermanyssus gallinae, D. americanus, Liponyssus bursa,* and *L. sylviarum.*	Mid-western and Western, U.S.A.
Venezuelan equine encephalitis.	Mosquitoes	*Aedes taeniorhynchus*	South America.
		Mansonia titillans	Trinidad, B.W.I.
		Anopheles neomaculipalpis	South America.
Japanese B encephalitis.	Mosquitoes	*Aedes chemulpoensis*	North China.
		Culex tritaeniorhynchus, C. fatigans, and *C. annulirostris.*	U.S.S.R. and much of the Far East.
		Culex pipiens, var. *pallens*	Sino-Japanese areas, Southwest Pacific Islands and Maritime Provinces of Siberia.
Russian spring-summer encephalitis.	Ticks	*Haemaphysalis concinna*	European U.S.S.R., Siberia, Far Eastern Maritime Provinces.
		H. japonica Dermacentor	Eastern Maritime Provinces.
		Ixodes persulcatus	U.S.S.R.

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Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
St. Louis encephalitis	Mites and mosquitoes	*Dermanyssus gallinae, Culex tarsalis, C. pipiens, C. quinquefasciatus, and Aedes dorsalis.*	U.S.A., principally Midwest.
Semliki Forest encephalitis.	Mosquitoes	*Aedes abnormalis*	Uganda.
West Nile encephalitis	Mosquitoes	*Culex antennatus, C. pipiens,* and *C. univittatus.*	Tropical Africa.
Poliomyelitis	Nonbiting flies and cockroaches.	*Musca* spp., other nonbiting flies and Blattidae.	World-wide.
		Spirochetes	
Endemic relapsing fever	Ticks	*Ornithodoros asperus*	Asia.
		O. crossi	Northern India.
		O. erraticus	Portugal, Spain, Tunisia.
		O. hermsi	Western U.S.A., British Columbia.
		O. marocanus	Morocco, Spain.
		O. moubata, and *O. savignyi*	Africa.
		O. parkeri	Western U.S.A..
		O. rudis	Central and South America, Mexico.
		O. talaje	Central and South America.
		O. tartakovskyi	U.S.S.R.
		O. tholozani	Iran, Iraq, Syria, Israel, Cyprus, and U.S.S.R.
		O. turicata	Mexico, Western U.S.A.
		O. verrucosus	Iran.
Epidemic relapsing fever	Body louse	*Pediculus humanus*	World-wide.
Tropical ulcer	Nonbiting flies	*Musca* spp *Sarcophaga* spp. and other filth flies.	Cosmopolitan in tropical regions of the world
Yaws	Flies	*Hippelates flavipes* *Musca domestica* complex	Caribbean Region. Africa and subtropical regions.